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(71) Applicant *(for all designated States except US)*: MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Hofgartenstrasse 8, D-80539 München (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CARUSO, Frank [AU/DE]; Golmer Fichten 32, 14476 Golm (DE). TRAU, Dieter [DE/CN]; Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (CN). MÖHWALD, Helmuth [DE/DE]; Dr.-Gebauer-Strasse 21, D-55411 Bingen (DE). RENNEBERG, Reinhard [DE/CN]; Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (CN).

(74) Agents: WEICKMANN, H. et al.; Kopernikusstrasse 9, D-81679 München (DE).

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(54) Title: ENCAPSULATION OF CRYSTALS VIA MULTILAYER COATINGS

(57) Abstract: The invention refers to a new process for preparing coated crystals by coating crystal template particles with alternating layers of oppositely charged polyelectrolytes and/or nanoparticles.

Encapsulation of crystals via multilayer coatings

Specification

The invention refers to a new process for preparing coated crystals by coating crystal template particles with alternating layers of oppositely charged polyelectrolytes and/or nanoparticles.

The area of thin film fabrication, in which ordered, functional supramolecular structures are the chief goal, has been greatly impacted by the recent introduction of the layer-by-layer (LbL) self-assembly technique (Decher, Science 1997, 277, 1232). The LbL method permits the fabrication of multilayer thin film assemblies on solid supports by the spontaneous sequential adsorption of oppositely charged species from dilute aqueous solutions onto charged substrates. The driving force for the multilayer film build-up is primarily due to the electrostatic attraction and complex formation between the charged species deposited. The LbL approach was initially employed to construct multilayer films of polyelectrolytes (Decher, Science 1997, 277, 1232), and subsequently extended to include proteins (Lvov et al, J. Am. Chem. Soc. 1995, 117, 6117; Onda et al, T. Biotech. Bioeng. 1996, 51, 163; Caruso et al, Langmuir 1997, 13, 3427), nucleic acids (Decher et al, J. Biosens. Bioelectron. 1994, 9, 677; Sukhorukov et al, Thin Solid Films 1996, 284/285, 220; Caruso et al, Anal. Chem. 1997, 69, 2043), dyes (Araki et al, Langmuir 1996, 12, 5393; Yoo et al, Synthetic Metals 1997, 85, 1425; Ariga et al, J. Am. Chem. Soc. 1997, 119, 2224), dendrimers (Tsukruk et al, Langmuir 1997, 13, 2171), and various inorganic nanoparticles (Kleinfeld et al, Science 1994, 265, 370; Keller et al, J. Am. Chem. Soc. 1994, 116, 8817; Kotov et al, J. Am. Chem. Soc. 1997, 119, 6821; Kotov et al, J. Phys. Chem. 1995, 99, 13065; Feldheim et al, J. Am. Chem. Soc. 1996, 118, 7640; Schmitt et al, Adv. Mater 1997, 9, 61; Lvov et al, Langmuir

1997, 13, 6195) in polyelectrolyte multilayer assemblies by replacing one of the polyions by a similarly charged species.

The vast majority of studies concerning the LbL technique have employed macroscopically flat charged surfaces as substrates for multilayer film formation. For example, U.S. 5,716,709 describes multilayered nanostructures comprising alternating organic and inorganic ionic layers on a flat substrate, such as a silicon wafer. Recently, Keller et al reported the preparation of alternating composite multilayers of exfoliated zirconium phosphate sheets and charged redox polymers on (3-aminopropyl)-triethoxysilane-modified silica particles (Keller et al, J. Am. Chem. Soc. 1995, 117, 12879).

In more recent studies (Caruso et al., Macromolecules, 1999, 32, 2317; Caruso et al., J. Phys. Chem. B.1998, 102, 2011; Sukhorukov et al., Colloids Surf. A: Physicochem.Eng.Aspects 1998, 137, 253), the LbL approach was successfully applied to utilise submicron- and micron-sized charged colloidal particles as the adsorbing substrates to produce colloid-supported polyelectrolyte multilayer films: regular step-wise polyelectrolyte multilayer growth was observed on the colloids.

Considerable scientific effort has focussed on the fabrication of composite micro- and nanoparticles that consist of either organic or inorganic cores coated with shells of different chemical composition (Kawahashi and Matijevic, J.Colloid Interface Sci. 1991, 143, 103; Garg and Matijevic, J.Colloid Interface Sci. 1988, 126; Kawahashi and Matijevic, J.Colloid Interface Sci. 1990, 138, 534; Ohmori and Matijevic, J.Colloid Interface Sci. 1992, 150, 594; Giersig et al., Adv. Mater. 1997, 9, 570; Liz-Marzan et al., Langmuir 1996, 12, 4329; Liz-Marzan et al., J.Chem.Soc.Chem.Commun. 1996, 731; Giersig et al., Ber.Bunsenges.Phys.Chem. 1997, 101, 1617; Correa-Duarte et al., Chem.Phys.Lett. 1998, 286, 497; Bamnolker et al., J.Mater.Sci.Lett. 1997,

16, 1412; Margel and Weisel, J.Polym.Sci.Chem.Ed. 1984, 22, 145; Philipse et al., Langmuir 1994, 10, 92). These core-shell particles often exhibit properties which are significantly different to those of the templated core (e.g. different surface chemical composition, increased stability, higher surface area, as well as different magnetic and optical properties), thus making them attractive both from a scientific and technological viewpoint. Applications for such particles are diverse, ranging from capsule agents for drug delivery, catalysis, coatings, composite materials, as well as for protecting senstitive agents such as enzymes and proteins. Previous investigations have demonstrated that polymeric microparticles and inorganic cores can be coated with uniform layers of various materials, including silica, yttrium basic carbonate, zirconium hydrous oxide, either by controlled surface precipitation reactions on the core particles, or by direct surface reactions.

U.S. Patent 5,091,187 discloses the coating of micro-crystals with phospholipids. EP-A-0 516 252 discloses the coating of nanocrystalline-magnetic particles with natural or synthetic glycosamino-glucans which may be selected from chondroitin-sulphate, dermatan-sulphate, heparan-sulphate, heparin and analogs thereof.

U.S. Patent 5,705,222 discloses a process for preparing composite particle dispersions wherein a plurality of core particles is dispersed in a first solution wherein the core particles do not irreversibly self-flocculate, an amount of polymer is added to the dispersion of core particles, wherein the polymer has an affinity for the dispersed core particles and the excess polymer is removed by a solid/liquid separation process, i.e. centrifugation or decanting.

In DE 198 12 083.4 it was suggested that soluble colloidal cores can be used as templates for the sequential deposition of polyelectrolytes to fabricate novel three-dimensional hollow polymer shells. From

PCT/EP99/01854 the preparation of coated capsules and hollow shells by coating colloidal particles with alternating layers of oppositely charged nanoparticles and polyelectrolytes is known. DE 199 075 52.2 discloses a process for preparing capsules having a polyelectrolyte shell wherein the template particles are selected from aggregates of biological and/or amphiphilic materials such as cells, cell aggregates, subcellular particles, virus particles and aggregates of biomolecules.

The coating of biocrystals, however, represents several challenges which are not applicable when templating other solid core particles including biological templates. First, the crystals are formed under delicate solution conditions, hence suitable conditions have to be found which facilitate polymer multilayer deposition on the crystal surface and do not destroy the crystal morphology (i. e. to avoid its solubilization), and second, the permeability of the polymer capsule walls must be such that it permits encapsulation of the bio-crystal. In addition, since the primary usefulness of biological molecules is their biological function, their activity should be preserved when encapsulated.

Herein we report sucessful coating of crystals, e. g. bio-crystals by depositing alternating layers of oppositely charged polyelectrolytes and/or nanoparticles on crystal template particles via the sequential electrostatic adsorption of polyelectrolyte and/or nanoparticles from solution. Functional biomolecules are obtainable after solubilization within the polyelectrolyte/nanoparticle shell and/or after removal of the shell.

Thus, a first aspect of the present invention is a process for preparing coated crystals comprising the steps:

(a) providing a dispersion of crystal template particles in a solvent and

(b) coating said particles with a multilayer comprising alternating layers of oppositely charged polyelectrolytes and/or nanoparticles.

Suitable crystal template particles are particles of crystallized substances. Preferably the crystals are particles of crystallized organic compounds and/or particles of crystallized biomolecules (bio-crystals) (including molecules which occur in living organisms and/or which influence the metabolism of living organisms). E. g. the crystals may be selected from crystals of proteins, peptides, nucleic acids, lipids, carbohydrates, saccharides, drugs etc., whereas protein crystals are preferred. Examples of protein crystals are crystals of antibodies, enzymes, such as oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases, virus capsid protein crystals, peptide crystals, S-layer protein crystals, glycoprotein crystals, receptor protein crystals and cytosolic protein crystals. The proteins may be natural, recombinant and/or synthetic proteins. Examples of nucleic acid crystals are crystalline DNA, crystalline RNA and crystalline oligonucleotides. Examples of crystalline low molecular weight materials are crystalline drugs, crystalline vitamins, crystalline nutrients, crystalline hormones and crystalline organic or inorganic salts.

It is also possible to use crystal template particles from crystalline bio-material, crystalline organic material, crystalline inorganic material or mixtures thereof. Examples of crystalline organic material are crystalline drugs, vitamins, nutrients, hormones, growth factors, pesticides and antibiotics.

The crystalline template particles may be derived from a single crystal or an amorphous crystal material. Suitable crystal template particles may be obtained, for example, from natural sources, may be prepared recombinantly, synthetically or by a cell culture or may be a PCR product.

Preferably, the template particles have an average diameter of up to 500 μm, in particular, $\leq$ 50 μm, more particularly $\leq$ 10 μm, more preferably $\leq$ 5 μm, and most preferably $\leq$ 2 μm. The minimum diameter of the template particles is preferably 10 nm, more preferably 100 nm, most preferably 200

nm and, in particular, 1 μm. The structure or shape of the template may be in any desired form, e. g. rectangular, square, spherical, triangular and various other forms depending on the structure of the crystal to be templated.

Important advantages of the crystal templating procedure presented are its versatility and generality. The thickness of the polymer walls can be controlled by varying the number of polyelectrolyte/nanoparticle deposition cycles, thus providing a straightforward and simple means to vary the permeability of the capsules. It can also be used to encapsulate biological materials in crystalline form in order to perform chemical reactions, e.g. enzymatic reactions. Furthermore, the entire volume of the capsule consists of an active biomolecule, making the activity per volume of the encapsulated biomolecule several orders of magnitude higher than that of conventional or immobilized biomolecules. This has the advantage of reducing the reaction times and the volume of biomolecule required, and maximizes activity and productivity. The multilayer coating also provides a way to preserve and/or prolong the activity of the encapsulated biomolecules by protecting them in environments where certain substances, e. g. macromolecules, degrading enzymes, e.g. proteases, nucleases, etc., microorganisms, inhibitors etc. may cause their degradation.

The successful encapsulation of bio-crystals by the nanoscale engineering of polymer capsules on bio-crystal templates as demonstrated here, coupled with the easy diffusion of small mobile substrates and reaction products through the polymer walls, is a promising way to both control and increase the efficiency of catalytic processes. Combining these templating process with recently developed techniques that allow the layering of various enzyme layers on colloids yields enzyme nanoreactor systems for sequential biocatalytic reactions. Columns packed with the biocrystal loaded capsules can be used for chromatographic separations and - in the case of encapsulated enzymes - as catalytic converters. Further, the introduction of

functionalities, e. g. magnetic properties, by incorporation of suitable nanoparticles in the polymer walls provides a multifunctional system with a plurality of properties. Together, these developments provide outstanding opportunities to produce tailored and optimized systems for numerous applications in the biotechnology area.

The process according to the present invention comprises coating the template particles with coatings of polyelectrolyte molecules and/or nanoparticles. The polyelectrolytes are usually polymers having ionically dissociable groups which may be a component or substituent of the polymer chain. Preferably, linear or/and water-soluble polyelectrolytes are used. However, polyelectrolytes being non-linear polymers or mixtures of linear and non-linear polymers also can be used. Depending on the type of dissociable group, polyelectrolytes are subdivided into polyacids and polybases. Upon dissociation polyacids separate protons to give polyanions. Examples of polyacids are polyphosphoric acid, polyvinyl or polystyrene sulphuric acid, polyvinyl or polystyrene sulfonic acid, polyvinyl or polystyrene phosphonic acid and polyacrylic acid. Examples of the respective salts, which are also called polysalts, are polyphosphate, polysulphate, polysulfonate, polyphosphonate and polyacrylate.

Polybases contain groups which are capable of accepting protons, e.g. by reacting with acids to give salts. Examples of polybases are polyamines, such as polyethylene amine, polyvinyl amine and polyvinyl pyridine or poly(ammonium salts), such as poly (diallyl dimethylammonium chloride). Polybases form polycations by accepting protons.

The polyelectrolytes may be or may be based on organic polymers, inorganic polymers and biopolymers or mixtures thereof. Preferred examples of organic polymers are biodegradable polymers such as polyglycolic acid (PGA), polylactic acid (PLA), polyamides, poly-2-hydroxy butyrate (PHB), polycaprolactone (PCL), poly(lactic-co-glycolic)acid (PLGA) and copolymers

thereof. Further preferred examples of polyelectrolytes are labelled polymers, e. g. fluorescently labelled polymers, conducting polymers, liquid crystal polymers, photoconducting polymers and photochromic polymers as well as copolymers thereof. Still further preferred examples of polyelectrolytes are sillafines, i.e. silicon containing cationic polyelectrolytes (cf. Science 286 (1999), 1129-1132).

Preferred examples of suitable biopolymers are poly amino acids such as peptides or S-layer proteins, poly carbohydrates such as dextrins, pectins, alginates, glycogens, amyloses or chitins, polynucleotides, e. g. DNA, RNA, oligonucleotides, gelatins (gelatin A, gelatin B) or modified biopolymers, e. g. carboxymethyl cellulose, carboxymethyl dextran or lignin sulfonates.

Preferred examples of suitable inorganic polymers, based on which the polyelectrolytes can be created, are polysilanes and silanoles, polyphosphazenes, polysulfazenes and polysulfides and polyphosphates.

The polyelectrolytes can be cross-linked after templating. The cross-linking can be done between the polymers in one layer or/and between polymers of different layers.

The nanoparticles preferably have an average diameter from 1 to 100 nm, more preferably from 5 to 50 nm and most preferably from 10 to 20 nm. The structure or shape of the nanoparticle may be in any desired form, with the most preferable shape being spherical and/or monodisperse.

The nanoparticles may be selected from inorganic, organic and biological materials. Preferred examples of inorganic particles are ceramic particles, e.g. oxidic ceramic particles, such as silicon dioxide, titanium dioxide, zirconium dioxide optionally doped with other metal oxides, magnetic particles such as iron oxide-containing particles such as Fe_3O_4, magneto-optical particles, nitridic ceramic particles, e.g. Si_3N_4, carbidic ceramic

particles, metallic particles, e.g. gold, silver, palladium and sulfur or selene-containing particles such as cadmium sulfide, cadmium selenide etc. Especially preferred are particles having magnetic properties. Examples of organic or biological nanoparticles are macromolecules, such as polypeptides, proteins, nucleic acids, enzymes, antibodies, receptor/ligand systems etc. and targeting molecules for therapeutical applications. Especially preferred biological nanoparticles are proteins, e.g. immunologically reactive proteins such as antigens and antibodies, e.g. IgG, or targeting molecules for therapeutical applications. Further, the biological nanoparticles may be deposited as conjugates containing a labelling group, e.g. a fluorescent group such as fluorescein. The resulting particles are suitable for analytical, e.g. immunological, detection methods. Further, an immobilization of enzymes, single enzymes or a plurality of enzymes, e.g. members of an enzymatic cascade, in single layers or different layers is of special interest because of the ability to increase the catalysis efficiency. Substrates could readily diffuse through the film and react with the immobilized enzyme, thereby producing product.

For the preparation of the coated particles according to the present application a dispersion of crystal template particles in suitable solvent is provided. A suitable solvent is a solvent where the crystal may be encapsulated. In some cases the solvent is an aqueous solvent. In other cases the solvent may be a mixture of an aqueous solvent with organic solvents. Still in other cases the solvent may be a pure organic solvent. Among the organic solvents polar solvents are preferred, e. g. polar protic solvents such as alcohols or polar aprotic solvents such as esters. Preferred examples of aqueous solvents are aqueous buffers which optionally contain detergents and/or antifoaming and/or antimicrobial agents. Aqueous solvents preferably contain buffer salts for pH and/or ionic strength adjustment. Preferred examples of mixed solvents are mixtures of water or aqueous buffers with polar protic organic solvents such as alcohols, organic acids, amines, pyridin or mercaptanes or mixtures of water or buffer with

polar aprotic organic solvents such as ketones, esters, ethers, ethyleneglycols, nitriles, formamide, alkylated formamides, nitroalkanes and dialkylsulfoxides. Preferred examples of organic solvents are polar protic organic solvents such as alcohols, organic acids, amines, pyridines, mercaptanes or mixtures thereof and polar aprotic organic solvents such as ketones, esters, ethers, ethyleneglycols, nitriles, formamide, alkylated formamides, nitroalkanes, dialkylsulfoxides and mixtures thereof. Of course also mixtures of protic and aprotic solvents may be used.

The dispersion of the template particles in the solvent may contain a salt, e.g. NaCl or KAc in a concentration which preferably ranges from 50 mmole/l to 1 mole/l. The solvent, pH, the temperature and the salt concentration in the dispersion are adjusted that the respective crystal template particles which are to be encapsulated are not substantially dissolved. Encapsulation temperatures of from 0°C to 10°C, e. g. 4°C are preferred. E. g., the encapsulation of catalase enzyme crystals may be carried out in an aqueous solution of 1 M potassium acetate, pH 5, 4°C. Alternating layers of oppositely charged components, i.e. polyelectrolyte molecules and/or nanoparticles, different types of nanoparticles or combinations thereof are then deposited on said template particles. The pH of the dispersion is adjusted in such a way that the molecules, e.g. polyelectrolyte molecules and nanoparticles, each have opposite total charges, thus facilitating their deposition via electrostatics. The thickness of the coating which is determined by the number of layers is preferably 2 to 1000 nm, with 2 to 40 and particularly 2 to 20, e.g. 3 to 10 coatings being applied. Suitably, each layer may be comprised of a single species of polyelectrolyte and/or nanoparticle or of a mixture comprising at least two polyelectrolyte and/or nanoparticle species. Further, for each layer different polyelectrolyte and/or nanoparticle species may be used.

After application of each layer the excessive molecules (e.g. polyelectrolyte and/or nanoparticle) which have not contributed to forming the layer are

preferably separated off before the next layer is applied. Such separation can be done according to any known method, particularly centrifugation, filtration and/or dialysis.

In a preferred embodiment of the invention the template particles are at first coated with several layers of oppositely charged cationic and anionic polyelectrolytes before the alternating layers of nanoparticles and polyelectrolyte or the alternating nanoparticle layers are applied. The coating of template particles with several layers of oppositely charged polyelectrolytes is described in DE 198 12 083.4 to which express reference is made. Preferably, the template particles are coated with at least two and up to six layers of oppositely charged cationic and anionic polyelectrolytes, e.g. with three layers. The outermost polyelectrolyte layer is preferably oppositely charged with regard to the polyelectrolyte/ nanoparticle to be deposited.

The thickness of the shell walls around the template particles can be readily controlled by varying the number of deposition cycles, whilst the shell size and shape are predetermined by the dimensions of the template particle employed. The thickness of the shell can vary in a wide range, e.g. from 2 to 1000 nm, particularly from 5 to 250 nm. The capsule thickness and permeability for controlled release of encapsulated material can be controlled by the number of layers, by the nature of the polyelectrolytes and/or nanoparticles used and, if desired, by an additional cross-linking step.

Excessive molecules, e.g. polyelectrolytes and/or nanoparticles which do not contribute to forming the layers may be separated after each templating step. The separation can be done, e.g. by centrifugation, filtration, dialysis or by sorting technologies regarding a special property, such as optical or magnetic properties. By the process of the invention the template crystals may be obtained encapsulated after the templating process.

It is possible to at least partially solubilize the encapsulated crystals after the coating has been completed. The solubilization can be carried out by adjustment of solvent, pH, temperature and/or salt conditions. The solubilized compounds, e. g. biomolecules substantially retain their functionality, e. g. biological activity after encapsulation and subsequent solubilization.

Further, it was found that the capsule walls can be ruptured or dissolved under conditions which do not significantly impair the activity of the encapsulated biomolecules (as crystals or after solubilization). The capsule walls may be dissolved by adjusting appropriate conditions, such as pH, e. g. under alkaline or acidic conditions, by means of chemical or biochemical degradation, or by physical methods, e. g. ultrasound.

Of particular importance for the use of the coated particles is the permeability of the shell wall. The permeability of the shell wall can be influenced by the selection of the polyelectrolytes used for the shell, the wall thickness and the ambient conditions. It is thus possible to selectively determine and change the permeability properties. Further, the permeability of the shell wall can be influenced by partially dissolving parts of the shell walls, e. g. by using a deproteinizer.

The permeability properties can be further modified by pores in at least one of the layers. Such pores can be formed by the polyelectrolytes or nanoparticles themselves if suitably chosen. By incorporating selective transport systems such as carriers or channels into the polyelectrolyte shell the transversal transport properties of the shell can be adapted to the respective application. The pores or channels of the shell wall can be selectively opened and closed, respectively, by chemically modifying and/or changing the ambient conditions. A high salt concentration of the medium used for the deposition of the polyelectrolyte results in a low packing density and a high permeability of the shell wall. On the other hand, a high

salt concentration of the medium used for the deposition of the nanoparticles results in a high packing density. Thus, by adjusting the salt concentrations in the deposition medium, the permeability of the shell can be controlled, as desired. Further, the permeability properties of the shell may be modified by selecting the conditions for decomposing the core, e.g. by selecting the temperature and heating conditions in a calcination procedure.

Still another method to modify the permeability of the shell walls is the deposition of one or several lipid layers on the outside of the shell wall or as an intermediate layer. Furthermore, the permeability may be modified by deposition of S-layer proteins. These proteins are able to build monolayers having a thickness of e. g. 5-20 nm by self-assembling on surfaces. These monolayers have a very defined structure and also a defined pore size of e. g. 4-8 nm. Incorporating such proteins in the capsule wall allows a further control of the capsule permeability properties.

Finally, the templated biomolecules can be at least partially disintegrated after the coating has been completed. They can be disintegrated in appropriate reagents, e. g. deproteinizer solutions or thermally (e.g. by calcination to temperatures of at least 500°C). After dissolution of the biomolecules hollow shells remain which are composed of polyeletrolyte material, nanoparticle material or optionally polyelectrolyte and nanoparticle material. The resulting hollow shells may be inorganic or organic shells or composite inorganic-organic or composite inorganic-inorganic shells depending on the method of core removal. For example, when thermal treatment (calcination) is used, all organic matter is removed, hence only inorganic shells are obtained. Exposure to deproteinizer solutions to remove the core results in polyelectrolyte shells or composite hollow particles in which the core is removed but the polyelectrolyte assembled between the nanoparticle layers remains in the shell.

The hollow shells may be characterized by any known methods, e.g. by scanning and transmission electron microscopy and atomic force microscopy. Preferably, the shells are uniform layers of regular thickness and can find applications in numerous areas, such as medicine, pharmaceutics, catalysis, optics, magnetics, separation and sensing. In the hollow shells of the present invention active agents, e.g. inorganic or/and organic substances can be encapsulated. Examples of active agents are pharmaceutic agents so that the shells can be used as drug delivery systems in order to transport the active agents to the desired place in the organism. Further, also contrasting agents can be enclosed in the shells, e.g. to improve the quality of images obtained by ultrasonic examination. It is also possible that the hollow shells themselves are used as contrasting agents.

A further aspect of the present invention is a coated crystal particle, preferably a bio-crystal particle having a core which is a template particle and a multilayer shell comprising alternating layers of oppositely charged inorganic polyelectrolytes and/or nanoparticles. Still a further aspect of the present invention is a coated particle having a core comprising an at least partially solubilized crystal template particle, preferably a bio-crystal particle and a multilayer shell comprising alternating layers of oppositely charged polyeletrolytes and/or nanoparticles. Preferably, the average diameter of the coated particle is 15 μm or less, more preferably 100 nm to 10 μm.

Still a further aspect of the present invention is a hollow shell obtainable by disintegrating the template biomolecules of the coated particle as described above. The hollow shell may be an organic structure, an inorganic structure or a composite organic-inorganic structure depending on the method used to remove the core.

The coated particle may be used as a system for targeted delivery and/or controlled release of encapsulated crystallized and/or solubilized substances.

Targeting may be accomplished by selecting specific nanoparticles and/or polyelectrolytes for the construction of the multilayer shell which provide specific functional properties allowing targeting of the resulting capsule. Examples of functional nanoparticles are nanoparticles which provide magnetic properties to the resulting capsules. Further examples of nanoparticles having targeting function are biomolecules such as specific immunoglobulins, receptor ligands etc. Controlled release of the encapsulated crystallized and/or solubilized substances is obtainable by choosing pH swellable, thermally or environmentally responsive and/or biodegradable polyelectrolyte and/or nanoparticle constituents of the capsule wall.

The coated particles may be used to prevent degradation of the encapsulated material, e.g. in the body of animals and humans. It can also be used to control the ability of the encapsulated material to permeate cell walls or bio-membranes. It further can be used to control the immune-reaction of an organism against the encapsulated material by the nature of the capsule material.

Further, the invention is illustrated by the following figures and examples:

Figure 1 is a scheme showing an embodiment of the process used to encapsulate biomolecules by using biocrystals as templates for the deposition of polymer multilayers. (1), (2) Polyelectrolyte layers are deposited step-wise onto the biocrystal templates by making use of the surface charge reversal that occurs upon adsorption of each layer. Each polyelectrolyte is removed by repeated centrifugation/wash cycles before the next layer is deposited. (3) Solubilisation of the biomolecule inside the polymer capsule by exposure to solutions of pH > 6 or acidic (approximately pH < 4) solutions in a morphology change of the polymer capsule. (4) Release of the biomolecule by

rupturing the polymer capsule may e. g. be achieved by exposure to solutions of pH > 11. (5) Exposure of the encapsulated biomolecule to an oxidising solution results in decomposition of the biomolecule which then is expelled from the interior through polymer walls, leaving behind hollow polymer capsules that originally encapsulated the biomolecule.

Figure 2 shows images of uncoated and polymer-multilayer coated catalase crystals. **a.** AFM image of an uncoated crystal showing its essentially square shape and micrometer dimensions. **b.** Fluorescence optical micrographs of catalase crystals coated with eight [(PSS/FITC-PAH)$_4$] polyelectrolyte multilayers showing the applicability of the coating process to enzyme crystal templates. The polycation, PAH, deposited in alternation with the polyanion, PSS, is fluorescently labelled with fluorescein isothiocyanate (FITC). No noticeable change in crystal morphology is observed with the polymer multilayer coating.

Figure 3 shows representative optical micrographs of the polymer capsules encapsulating solubilised enzyme. **a.** A polymer capsule containing solubilized enzyme in its interior. The essentially spherical shape is assumed upon solubilisation of the enzyme as a result of exposure to solutions of pH > 6. Solubilization also occurs upon exposure to solutions of pH < 4. **b.** Rupturing of a polymer capsule causes the release of the entrapped, solubilized enzyme. Rupturing is achieved by subjecting the polymer capsules to alkaline solutions of pH > 11.

Figure 4 shows micrographs of air-dried hollow polymer capsules obtained after decomposition of the encapsulated enzyme. **a.**

AFM image of a polymer capsule comprising eight [(PSS/PAH)$_4$] polyelectrolyte layers. The polymer capsule spreads out on the glass surface on which it is dried and folds and creases in the shell can be seen. Some undecomposed enzyme can still be seen in the interior of the capsule. The z range (height) of the image is 650 nm. **b.** TEM image also shows folds and creases as well as the presence of a small amount of residual enzyme inside the capsule.

Figure 5 shows the stability of (a, d, e) solution solubilized catalase and (b, c) polymer multilayer-encapsulated (solubilized) catalase with respect to proteolysis. (a) Solution solubilized catalase crystals, no protease incubation (control); (b) [(PSS/PAH)$_2$]-coated (4 layers) catalase, protease incubation; (c) [(PSS/PAH)$_4$]-coated (8 layers) catalase, protease incubation; (d) and (e) repeat experiments for solubilized catalase, protease incubation. Proteolysis of the catalase was determined by measuring the decrease in the catalase enzyme activity.

1. Methods

1.1 Catalase crystal encapsulation

The catalase enzyme crystals (Sigma, C-100) were washed twice with a chilled (4°C) solution of 1 M potassium acetate at pH 5 (buffer), with intermittent centrifugation steps (500 g, 4 min, 4°C) to remove the supernatant, prior to polymer coating. The polymer layers were assembled onto the enzyme crystals by the alternate depostion of poly(sodium 4-styrenesulfonate) (PSS), M_w 70,000, and poly(allylamine hydrochloride) (PAH), M_w 8,000 - 11,000, both obtained from Aldrich. PSS was dialyzed against water and lyophilized before use. The first layer was deposited by

adding a 0.5 ml aliquot of a 5 mg/ml aqueous PSS solution (containing 1 M potassium acetate, pH 5, 4°C) to 0.2 ml of the crystal suspension, occasionally shaking the suspension, and allowing 25 min for adsorption. The excess polyelectrolyte was removed by 3 repeated centrifugation (500 g, 4 min, 4°C)/chilled buffer wash/redispersion cycles. The next layer, PAH, was deposited from a 5 mg/ml solution containing 1 M potassium acetate (pH 5 at 4°C) using the same procedure and conditions. Subsequent alternating PSS and PAH layers were deposited in identical fashion until the desired number of polymer multilayers was achieved. Hollow polymer capsules were obtained by exposing the polymer multilayer-coated capsules to a deproteinizer solution (Medical Instruments). The deproteinizer destroys the protein.

1.2 Determination of catalase activity

The enzyme was released from the polymer capsules by exposure to ultrasound for 5 min at 20°C in 1 M phosphate buffer at pH 7 (this caused rupturing of the polymer walls). The catalase activity was determined using a standard enzyme activity assay (Biochemica Information, 15-16 Boehringer Mannheim (1987); Tijsen, Practice and Theory of Enzyme Immunoassays, 8th Impression (1993), Elsevier, 202-203): that is, by measuring the decomposition of the substrate hydrogen peroxide (H_2O_2) at 240 nm. The concentration of the catalase was determined spectrophotometrically at 280 nm and 405 nm (soret band). The specific acitvity of the uncoated catalase was measured in the same way and used for comparison.

1.3 Electrophoretic mobility and microscopy measurements

Electrophoretic mobilities of the uncoated and polymer-multilayer coated enzyme crystals were measured using a Malvern Zetasizer 4 as described elsewhere (Caruso et al., J. Phys. Chem. B 102, 2011 - 2116 (1998);

Sukhorukov, G. B. et al., Polym. Adv. Technol. 9, 759 - 767 (1998); Donath et al., Angew. Chem. Int. Ed. 37, 2201 - 2205 (1998). The mobility u was converted into a ξ-potential using the Smoluchowski relation $\xi = u\eta/\epsilon$, where η and ϵ are the viscosity and permittivity of the solution, respectively. Atomic force microscopy (AFM) images were obtained using a Digital Instruments Nanoscope IIIa AFM in tapping mode (TM) on samples deposited onto cleaned glass slides and air-dried. Samples for transmission electron microscopy (TEM) were prepared by deposition of aqueous solutions of the hollow polymer capsules upon a carbon-coated copper grid, allowing them to air dry for one minute, and then blotting off the extra solution.

1.4 Proteolysis Experiment

10 mg of protease (Streptomyces griseus; Sigma P6911; activity = 5.2 U/mg solid) was dissolved in 500 µl of phosphate buffered saline (PBS, pH 7.0)(100U/ml) and used as the stock solution. 200 µl samples of the polyelectrolyte-coated catalase and solubilized catalase where separately incubated in a water bath at 37°C with either 30 µl of the stock protease solution of 30 µl of PBS (control experiment). The starting activity of the catalase for all samples was approximately 12000 U/ml catalase. The starting activities were normalized to 100%. The final protease activity in the sample was 13000 U/ml. Proteolysis of the catalase was determined by measuring the decrease in the catalase enzyme activity. The catalase activity measurements where carried out according to the previously described method (i.e. by spectrophotometric detection (240 nm) of the decomposition of hydrogen peroxide, see Tijsen, Practice and Theory of Enzyme Immunoassays, Elsevier, 8th Impression (1993), pp. 202 - 203.15). 5 µl samples were diluted and used for the activity measurements.

2. Results

The basic approach using enzyme crystals as templates for encapsulation by polymer multilayers is illustrated in Fig. 1. The method takes advantage of the fact that catalase is a crystalline suspension in water at pH 5 - 6 and therefore can be treated as a colloidal particle. The catalase crystals exhibit a positive surface charge (Zeta potential = +20 mV) in water at pH 5 (catalase isoelectric point = 5.8), as determined by electrophoretic mobility measurements.

A tapping mode atomic force microscopy (AFM) image of a catalase crystal, approximately 8 x 12 μm in size, is shown in Fig. 2(a). Next, the first polyelectrolyte layer, poly(styrenesulfonate) (PSS), which bears an opposite charge to the positively charged crystal surface, was added to the crystal suspension and allowed to self-assemble onto the crystal (Fig. 1). At this stage the enzyme crystal surface charge was reversed to a Zeta-potential of -30 mV, indicating the successful adsorption of PSS. Repeating alternating depositions of poly(allylaminehydrochloride) (PAH) and PSS produced crystals with alternating positive (+20 mV) and negative (-30 mV) surface potentials, respectively. These data clearly demonstrate recharging the surface.

In order to verify that the enzyme crystals are sucessfully coated with polymer multilayer shells, a fluorescently labelled polyelectrolyte (fluorescein isothiocyanate - (FITC) - PAH) was substituted for each alternate PAH deposition in the build-up process. The fluorescence, as seen under a fluorescence microscope, originates only from the surface of the polymer coated enzyme crystals (Fig 2b). The fluorescence signal was found to increase systematically with an increasing number of fluorescent layers. No noticeable change in size or shape of the crystals were observed with the polymer multilayer shell coating. Further, the polymer-coated enzymes could be stored for at least 30 days at 4°C without any noticeable change in

morphology. These results show that enzyme crystals can be encapsulated within a polymer multilayer capsule via the step-wise and regular assembly of oppositely charged polymers, and that the crystal size and shape is retained upon their coating.

Exposure of the polymer multilayer-encapsulated enzyme crystals to a solution of pH < 4 and pH > 6 resulted in the polymer capsule assuming a more spherical shape (Fig. 3a). Similar morphology changes were observed for other coated crystals, although perfectly spherical capsules were not always observed (as seen in Fig. 3a). The morphology change of the polymer capsules at pH < 4 and pH > 6 is attributed to the solubilization of the enzyme crystal, which occurs because the polymer multilayers are permeable to the mobile ions in the solution phase. The capsules did not rupture solely as a result of enzyme solubilization, indicating their high stability.

Evidence that the enzyme was encapsulated within the polymer capsules was obtained by deliberately rupturing the polymer shells. The polymer multilayer capsules were found to partially rupture upon exposure to alkaline solutions of pH > 11. An optical micrograph image of shell rupture is displayed in Fig. 3b. Soon after shell wall rupture, solubilized enzyme is expelled from the interior of the capsule. The expulsion of the enzyme is caused by the osmotic pressure between the interior and exterior of the polymer capsules. Video microscopy verifies that the solubilized enzyme is expelled upon exposure to solutions of pH > 11 as a result of capsule rupture. These data confirm that the solubilized enzyme is entrapped within the polymer capsules.

The catalase activity was measured after its solubilization and release from the polymer capsules. A recovered specific activity of 97% was obtained, compared with 100% for the uncoated catalase. This shows that the

polymer coating of the catalase crystals proceeds without causing any significant loss of enzyme activity.

Fig. 4 shows representative AFM and TEM images of the air-dried polymer capsules, obtained after exposing the polymer-coated enzyme crystals to an oxidizing solution (deproteinizer). The enzymes are decomposed by the deproteinizing treatment, allowing the expulsion of their fragment constituents from the interior by permeating the polymer capsule walls. The polymer multilayer capsules remain intact, as can be seen in the images. The drying process (evaporation of the aqueous content by air-drying) induces a number of folds and creases in the polymer capsules. The shells are also flattened and some spreading is noticed; the diameters of the dried polymer capsules are approximately 20 μm. The texture of the shells is characteristic of the polyelectrolyte film, although some residual catalase can be seen in the capsules. From the AFM image for the polymer capsule, it can be deduced from the lowest height dimensions (35 nm), equivalent to twice the polymer capsule wall thickness, that the average thickness per polyelectrolyte layer is approximately 2 nm (the enzyme crystal templates were coated with eight layers). Cross-section TEM images and confocal microscopy show that hydrated polymer capsules maintain their spherical shape in solution, although spreading and creasing is observed on drying, as seen here after removal of the enzyme and subsequent drying.

The results of the proteolysis experiments are shown in Figure 5. Solubilized, uncoated catalase (curves d and e) was inactivated by protease to more then 90 % during an incubation time of 100 min. In contrast, no measurable loss in enzyme activity was observed for the polymer-encapsulated (solubilized) catalase within 100 min under the same conditions (curves b and c). (The catalase was solubilized and retained within the polymer multilayer capsule at the experimental pH of 7). Further experiments showed that the activity of the encapsulated enzyme was fully retained after 24 hours exposure to protease. These results clearly

demonstrate that a thin polymer coating of 4 layers (thickness of ca. 8 nm) is sufficient to prevent proteolysis of the polymer-encapsulated catalase.

Claims

1. A process for preparing coated crystals comprising the steps:
   (a) providing a dispersion of crystal template particles in a solvent and
   (b) coating said particles with a multilayer comprising alternating layers of oppositely charged polyelectrolytes and/or nanoparticles.

2. The process of claim 1, wherein said crystal template particles are bio-crystals.

3. The process of claim 1 or 2, wherein said crystal particles are protein crystals, peptide crystals, nucleic acid crystals, lipid based crystals, carbohydrate crystals or crystals from low molecular weight materials.

4. The process of claim 3, wherein said protein crystals are selected from antibody crystals, enzyme crystals, virus capsid protein crystals, S-layer protein crystals, glycoprotein crystals, receptor protein crystals and cytosolic protein crystals.

5. The process of claim 1, wherein said crystal template particles are selected from the group consisting of crystalline bio-material, crystalline organic material, crystalline inorganic material or mixtures thereof.

6. The process of claim 5, wherein the crystalline or organic material is selected from crystalline drugs, crystalline vitamins, crystalline nutrients, crystalline hormones, crystalline growth factors, crystalline pesticides and crystalline antibiotics.

7. The process of any one of claims 1 to 6, wherein the crystal template is a single crystal material or an amorphous crystal material.

8. The process of any of claims 1 to 7, wherein said template particles have an average diameter of 500 μm or less.

9. The process of claim 8, wherein said template particles have an average diameter of 50 μm or less.

10. The process of any one of claims 1 to 9, wherein said polyelectrolytes are linear molecules.

11. The process of any one of claims 1 to 10, wherein said polyelectrolytes are selected from inorganic, organic and biological polyelectrolytes and mixtures thereof.

12. The process of claim 10, wherein the organic polyelectrolyte is a polymer selected from biodegradable polymers, fluorescently labelled polymers, conducting polymers, liquid crystal polymers, photoconducting polymers, photochromic polymers, and copolymers and/or mixtures thereof.

13. The process of claim 10, wherein the biological polyelectrolyte is a polymer selected from polyamino acids, polycarbohydrates, polynucleotides and modified biopolymers.

14. The process of claim 10, wherein the inorganic polyelectrolyte is a polymer based on polysilanes, polysilanoles, polyphosphazanes, polysulfazenes, polysulfides and polyphosphates.

15. The process of any one of claims 1 to 14, wherein said nanoparticles have an average diameter of from 1 to 100 nm.

16. The process of any one of claims 1 to 15, wherein said nanoparticles are selected from inorganic, organic and biological particles or mixtures thereof.

17. The process of claim 16, wherein said nanoparticles are selected from particles which provide targeting properties.

18. The process of claim 16 or 17, wherein said nanoparticles are particles having magnetic properties.

19. The process of claim 16 or 17, wherein said nanoparticles are immunoglobulins or receptor ligands.

20. The process of any one of claims 16 to 19, wherein the inorganic nanoparticles are ceramic particles, magnetic particles, magneto-optical particles, nitridic ceramic particles, carbidic ceramic particles, metallic particles, and/or sulfur or selenium-containing particles.

21. The process of any one of claims 16 to 19, wherein the organic or biological nanoparticles are macromolecules and/or targeting molecules.

22. The process of any one of the preceding claims, wherein said solvent is selected from aqueous solvents, organic solvents and mixed aqueous/organic solvents.

23. The process of any one of the preceding claims further comprising the step:

(c) at least partially solubilizing the encapsulated crystals.

24. The process of claim 23, wherein said solubilization is carried out by adjustment of solvent, pH, temperature and/or salt conditions.

25. The process of any one of the preceding claims further comprising the step:

(d) rupturing the polyelectrolyte/nanoparticle shell.

26. The process of any one of the claims 1 to 24 further comprising the step:

(e) at least partially disintegrating encapsulated biomolecules.

27. Coated particle having a core which is a crystal template particle and a multilayer shell comprising alternating layers of oppositely charged polyelectrolytes and/or nanoparticles.

28. Coated particle having a core comprising an at least partially solubilized crystal template particle and a multilayer shell comprising alternating layers of oppositely charged nanoparticles and/or polyelectrolytes.

29. The particle of claim 27 or 28 having an average diameter of 50 μm or less.

30. Hollow shell obtainable by disintegrating the template particle of the coated particle of claim 27, 28 or 29.

31. Use of the particle according to any one of claims 27 to 29 as a system for targeted delivery and/or controlled release of crystallizable biomolecules.

Fig. 1

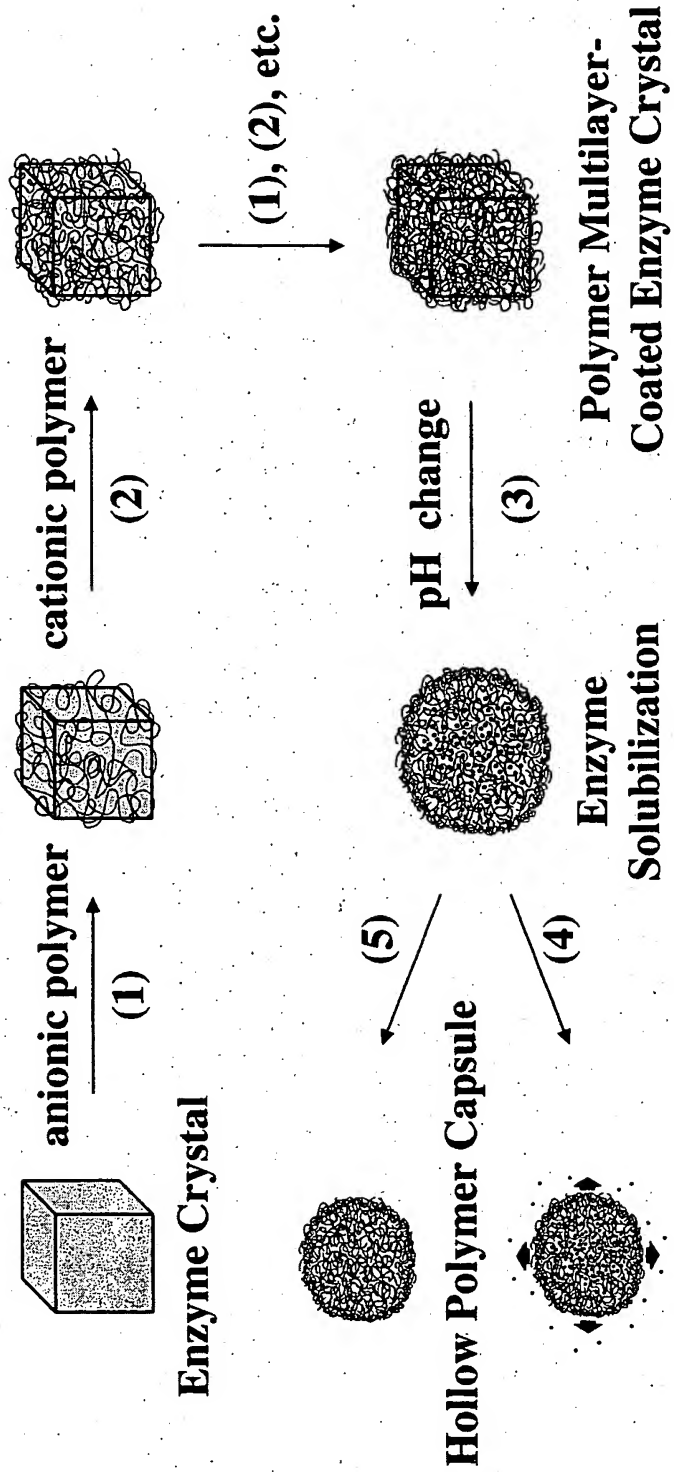

anionic polymer
(1)
Enzyme Crystal
cationic polymer
(2)
(1), (2), etc.
Polymer Multilayer-
Coated Enzyme Crystal
pH change
(3)
Enzyme
Solubilization
(5)
(4)
Hollow Polymer Capsule
Enzyme Release

Fig. 2

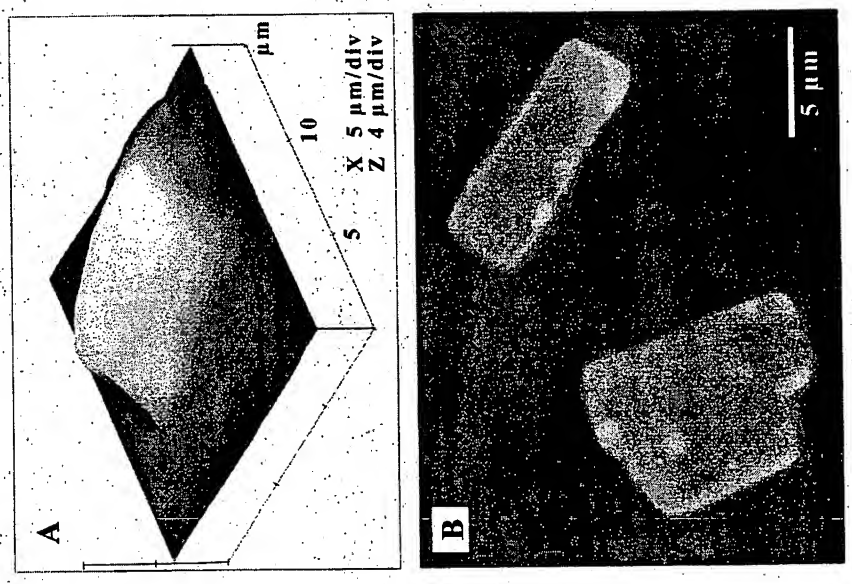


Fig. 3

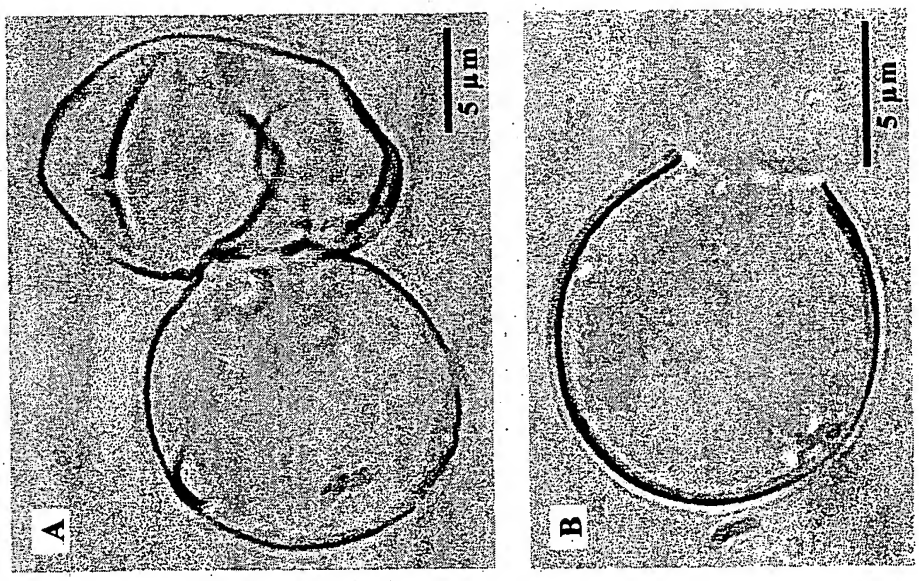


Fig. 4

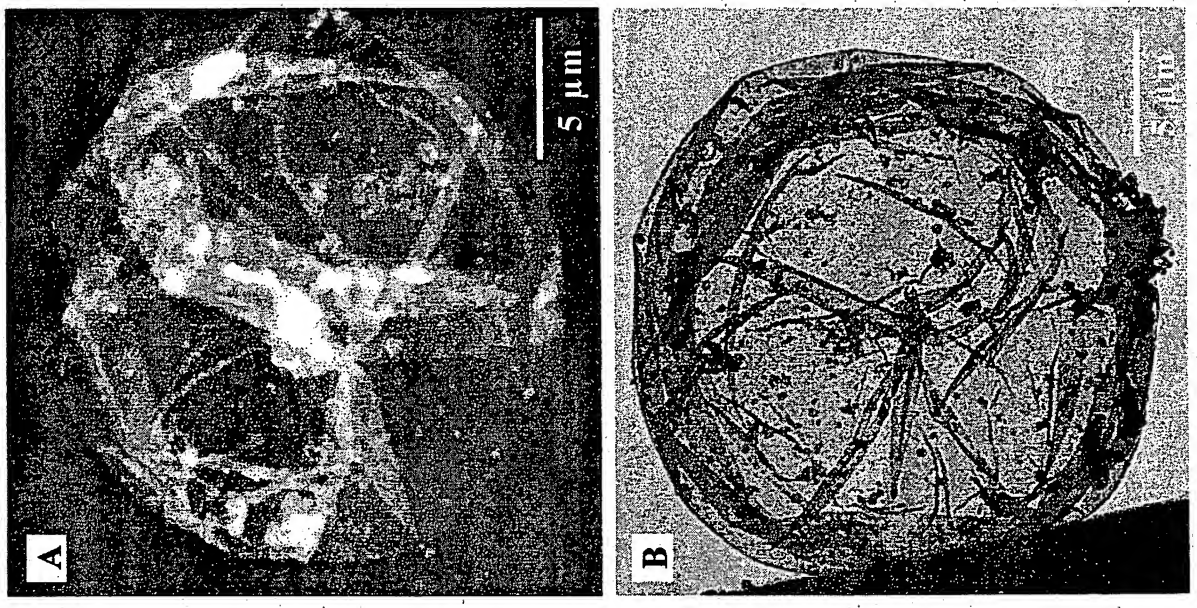
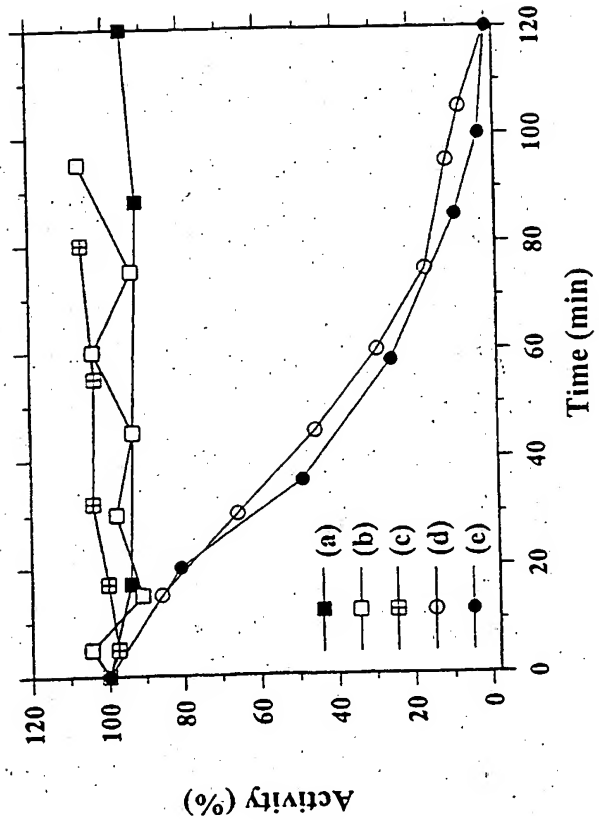


Fig. 5


Activity (%)
120
100
80
60
40
20
0
Time (min)
0
20
40
60
80
100
120
(a)
(b)
(c)
(d)
(e)

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
20077P WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 05358	09/06/2000	10/06/1999

Applicant

MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSEN.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 00/05358

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C30B29/58 A61K9/50 C12N11/04 C30B33/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C30B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 091 187 A (HAYNES DUNCAN H) 25 February 1992 (1992-02-25) cited in the application column 7, line 48 -column 8, line 66; figures 1,2A,2B; example 2	1,5-11, 22,27,29
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 September 2000	12/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cook, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat[illegible] Application No
PCT/EP 00/05358

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat[illegible]pplication No
PCT/EP 00/05358

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		WO 9602383 A	01-02-1996

PATENT COOPERATION TREATY

MD

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

WEICKMANN, H. et al
WEICKMANN & WEICKMANN
Patentanwälte
Kopernikusstrasse 9
81679 MÜNCHEN
ALLEMAGNE

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	22.06.2001
Applicant's or agent's file reference: 20077P WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/05358	09/06/2000	10/06/1999

Applicant
MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSEN..

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Krage, D Tel.+49 89 2399-7530

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
20077P WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/05358	09/06/2000	10/06/1999
International Patent Classification (IPC) or national classification and IPC		
C30B29/58		
Applicant		
MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSEN..		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/11/2000	22.06.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Kiliaan, S Telephone No. +49 89 2399 8446

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/05358

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-23 as originally filed

Claims, No.:

1-31 as originally filed

Drawings, sheets:

1/5-5/5 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-31
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-31
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-31
	No:	Claims

2. Citations and explanations
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:
   D2= EP-A-0 516 252 (DIAGNOSTIKFORSCHUNG INST) 2 December 1992 (1992-12-02) cited in the application.

2. Document D2 is considered to be the closest prior art. D2 discloses the coating of nanocrystalline magnetic particles consisting of a magnetic iron oxide nucleus of Fe_3O_4, Fe_2O_3 or their mixtures and an envelope consisting of natural or synthetic glycosaminoglycanes and/or their derivates chemisorbed to the particles shell surrounding the nucleus.
   The subject-matter of independent claim 1 differs from D2 in that the method of the invention comprises a step of coating the particles with a multilayer of alternating layers of oppositely charged polyelectrolytes and/or nanoparticles.
   Furthermore, the subject-matter of independent claims 27, 28, 30 (product) and 31 (use) is not disclosed in D2.
   Consequently, the subject-matter of independent claims 1 and 27, 28, 30 and 31 is considered to be new.

3. The method as disclosed in D2 is based on the chemisorption of glycosaminoglycanes resulting in a single layer of glycosaminoglycanes, whereas the method of the present invention is based on coulomb interactions between oppositely charged polyelectrolytes and/or nanoparticles resulting in a multilayer.
   The method of the present invention has the advantage that by varying the number of polyelectrolyte/nanoparticle deposition cycles the permeability can be changed very easily.
   Consequently, the subject-matter of claims 1, 27, 28, 30 and 31 is considered to involve an inventive step.

4. Claims 2 to 26 are dependent on claim 1 and claim 29 is dependent on claim 27 or 28. These claims as such also meet the requirements of the PCT with respect to novelty and inventive step.

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PATENT COOPERATION TREATY

PCT

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(PCT Article 36 and Rule 70)

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	No:	Claims	

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(71) Applicant *(for all designated States except US)*: MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Hofgartenstrasse 2, D-80539 München (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CARUSO, Frank [AU/DE]; Roennebergstrasse 6, D-12161 Berlin (DE). CARUSO, Rachel, Anne [AU/DE]; Roennebergstrasse 6, D-12161 Berlin (DE). DONATH, Edwin [DE/DE]; Dreetzer Strasse 1, D-16845 Giesenhorst (DE). MÖHWALD, Helmuth [DE/DE]; Dr.-Gebauer-Strasse 21, D-55411 Bingen (DE). SUKHORUKOV, Gleb [RU/RU]; Pustchino, Microraion AB 533, Moscow District, 142292 (RU).

(74) Agents: WEICKMANN, H. et al.; Kopernikusstrasse 9, D-81679 München (DE).

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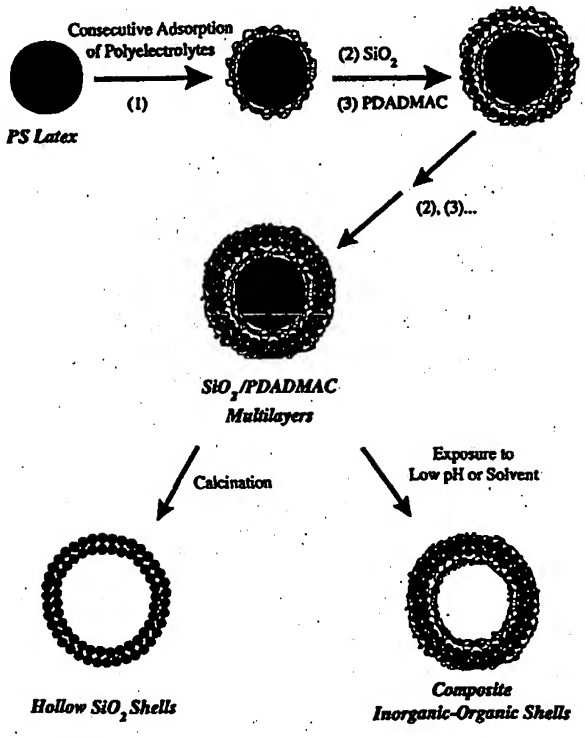
(54) Title: FABRICATION OF MULTILAYER-COATED PARTICLES AND HOLLOW SHELLS VIA ELECTROSTATIC SELF-ASSEMBLY OF NANOCOMPOSITE MULTILAYERS ON DECOMPOSABLE COLLOIDAL TEMPLATES

(57) Abstract

The invention refers to a new process for preparing coated particles and hollow shells by coating colloidal particles with alternating layers of oppositely charged nanoparticles and polyelectrolytes and optionally removing the colloidal cores.



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Fabrication of multilayer-coated particles and hollow shells via electrostatic self-assembly of nanocomposite multilayers and decomposable colloidal templates

Specification

The invention refers to a new process for preparing coated capsules and hollow shells by coating colloidal particles with alternating layers of oppositely charged nanoparticles and polyelectrolytes.

The area of thin film fabrication, in which ordered, functional supramolecular structures are the chief goal, has been greatly impacted by the recent introduction of the layer-by-layer (LbL) self-assembly technique (Decher, Science 1997, 277, 1232). The LbL method permits the fabrication of multilayer thin film assemblies on solid supports by the spontaneous sequential adsorption of oppositely charged species from dilute aqueous solutions onto charged substrates. The driving force for the multilayer film build-up is primarily due to the electrostatic attraction and complex formation between the charged species deposited. The LbL approach was initially employed to construct multilayer films of polyelectrolytes (Decher, Science 1997, 277, 1232), and subsequently extended to include proteins (Lvov et al, J. Am. Chem. Soc. 1995, 117, 6117; Onda et al, T. Biotech. Bioeng. 1996, 51, 163; Caruso et al, Langmuir 1997, 13, 3427), nucleic acids (Decher et al, J. Biosens. Bioelectron. 1994, 9, 677; Sukhorukov et al, Thin Solid Films 1996, 284/285, 220; Caruso et al, Anal. Chem. 1997, 69, 2043), dyes (Araki et al, Langmuir 1996, 12, 5393; Yoo et al, Synthetic Metals 1997, 85, 1425; Ariga et al, J. Am. Chem. Soc. 1997, 119, 2224), dendrimers (Tsukruk et al, Langmuir 1997, 13, 2171), and various inorganic nanoparticles (Kleinfeld et al, Science 1994, 265, 370; Keller et al, J. Am. Chem. Soc. 1994, 116, 8817; Kotov et al, J. Am. Chem. Soc. 1997, 119, 6821; Kotov et al, J.

Phys. Chem. 1995, 99, 13065; Feldheim et al, J. Am. Chem. Soc. 1996, 118, 7640; Schmitt t al, Adv. Mater 1997, 9, 61; Lv v et al, Langmuir 1997, 13, 6195) in polyelectrolyte multilayer assemblies by replacing one of the polyions by a similarly charged species.

The vast majority of studies concerning the LbL technique have employed macroscopically flat charged surfaces as substrates for multilayer film formation. For example, U.S. 5,716,709 describes multilayered nanostructures comprising alternating organic and inorganic ionic layers on a flat substrate, such as a silicon wafer. Recently, Keller et al reported the preparation of alternating composite multilayers of exfoliated zirconium phosphate sheets and charged redox polymers on (3-aminopropyl)-triethoxysilane-modified silica particles (Keller et al, J. Am. Chem. Soc. 1995, 117, 12879).

In more recent studies (Caruso et al, J. Phys. Chem. B.1998, 102, 2011; Sukhorukov et al., Colloids Surf. A: Physicochem.Eng.Aspects 1998, 137, 253), the LbL approach was successfully applied to utilise submicron- and micron-sized charged colloidal particles as the adsorbing substrates to produce colloid-supported polyelectrolyte multilayer films: regular step-wise polyelectrolyte multilayer growth was observed on the colloids.

Considerable scientific effort has focussed on the fabrication of composite micro- and nanoparticles that consist of either organic or inorganic cores coated with shells of different chemical composition (Kawahashi and Matijevic, J.Colloid Interface Sci. 1991, 143, 103; Garg and Matijevic, J.Colloid Interface Sci. 1988, 126; Kawahashi and Matijevic, J.Colloid Interface Sci. 1990, 138, 534; Ohmori and Matijevic, J.Colloid Interface Sci. 1992, 150, 594; Giersig et al., Adv. Mater. 1997, 9, 570; Liz-Marzan et al., Langmuir 1996, 12, 4329; Liz-Marzan et al., J.Chem.Soc.Chem.Commun. 1996, 731; Giersig et al., Ber.Bunsenges.Phys.Chem. 1997, 101, 1617; Correa-Duarte et al.,

Chem.Phys.Lett. 1998, 286, 497; Bamnolker et al., J.Mater.Sci.Lett. 1997, 16, 1412; Margel and Weisel, J.Polym.Sci.Chem.Ed. 1984, 22, 145; Philips et al., Langmuir 1994, 10, 92). These core-shell particles oft n exhibit properties which are significantly different to those of the templated core (e.g. different surface chemical composition, increased stability, higher surface area, as well as different magnetic and optical properties), thus making them attractive both from a scientific and technological viewpoint. Applications for such particles are diverse, ranging from capsule agents for drug delivery, catalysis, coatings, composite materials, as well as for protecting senstitive agents such as enzymes and proteins. Previous investigations have demonstrated that polymeric microparticles and inorganic cores can be coated with uniform layers of various materials, including silica, yttrium basic carbonate, zirconium hydrous oxide, either by controlled surface precipitation reactions on the core particles, or by direct surface reactions.

U.S. Patent 5,705,222 discloses a process for preparing composite particle dispersions wherein a plurality of core particles is dispersed in a first solution wherein the core particles do not irreversibly self-flocculate, an amount of polymer is added to the dispersion of core particles, wherein the polymer has an affinity for the dispersed core particles and the excess polymer is removed by a solid/liquid separation process, i.e. centrifugation or decanting.

An important extension of core-shell particles is the subsequent removal of the core, resulting in hollow particles or shells. Removal of the templated core has previously been achieved by calcining the coated particles at elevated temperatures or by chemical reactions causing dissolution of the core material. Hollow, submicron sized shells of yttrium compounds have been produced (Kawahashi and Matijevic, 1991, supra) by coating cationic polystyrene latex with yttrium basic carbonate and subsequently calcining. More recently, silica shells were generated by seeded polymerization of

tetraethoxysilane on the surface of polystyrene particles, followed by calcination (Bamnolker et al., 1997, supra). Using a similar method, monodisperse, hollow silica nanoparticles have b en produced by silica-coating gold nanoparticles, and by chemically dissolving the cores (Giersig et al., Ber.Bunsenges.Phys.Chem., 1997, supra). Hollow particles represent a special class of materials: their lower density and optical properties make them of interest in the fields of medicine, pharmaceutics, materials science and the paint industry.

Conventional methods for the preparation of coated nanoparticles or hollow nanoshells, however, have several disadvantages, since in many cases the formation of uniform and smooth layer structures having sufficient particle coverage as well as control of thickness is very difficult to achieve.

Further, it was suggested (DE 198 12 083.4) that the use of soluble colloidal cores as templates for the sequential deposition of polyelectrolytes can be used to fabricate novel three-dimensional hollow polymer shells.

Herein we report the construction of composite multilayers of nanoparticles and an oppositely charged polyelectrolyte on submicron-sized colloidal particles via the sequential electrostatic adsorption of nanoparticles and polyelectrolyte from dilute solution. Alternating nanoparticle-polyelectrolyte multilayers with various thicknesses have been fabricated. Further, a novel and yet simple method for the fabrication of submicron-sized, hollow, inorganic or composite organic-inorganic particles via colloid templated electrostatic LBL self-assembly of nanoparticle-polymer multilayers, followed by removal of the templated core and optionally the polymer used in the assembly process is presented.

Thus, a first aspect of the present invention is a process for preparing coated particles comprising the steps:

(a) providing template particles and

(b) coating said template particles with a multilayer comprising (i) alternating layers of oppositely charged nanoparticles and polyelectrolytes and/or (ii) alternating layers of oppositely charged nanoparticles.

Preferably, the template particles have an average diameter of up to 10 μm, more preferably $\leq$ 5 μm, and most preferably $\leq$ 2 μm. The minimum diameter of the template particles is preferably 10 nm, more preferably 100 nm, and most preferably 200 nm.

Suitable template particles may be selected from organic particles, inorganic particles, or any combination thereof. For example, the template particles may be inorganic particles including inorganic structures. In a preferred embodiment, the template particles are selected from organic polymer latices, such as polystyrene or styrene copolymer latices. On the other hand, also partially cross-linked melamine-formaldehyde template particles can be used which can be disintegrated under mild conditions, e.g. by adjusting the pH value to an acid value of e.g. $\leq$ 1.5, by dissolving in mild organic solvents such as DMSO or by chemical reactions, e.g. sulfonation with alkali sulfites, alkali hydrogen sulfites etc. Regarding the preparation of partially cross-linked melamine-formaldehyde template particles reference is made to DE 198 12 083.4, particularly Example 1, where it is described that in the polycondensation of melamine-formaldehyde precondensates (cf. DD 224 602) the polycondensation process can be interrupted after some time, e.g. 1 min to 1 h after start of the reaction, so that dissoluble, partially cross-linked melamine-formaldehyde template particles are obtained.

Other organic template particles, e.g. polystyrene latices, can be disintegrated by dissolving in appropriate organic solvents such as THF or by heating, e.g. to temperatures of 500°C or greater.

Th process according to the present invention comprises coating the template particles with alternating coatings of polyelectrolyte molecules and nanoparticles. The polyel ctrolytes ar usually polymers having ionically dissociable groups which may be a component or substituent of the polymer chain. Preferably, linear or/and water-soluble polyelectrolytes are used. Depending on the type of dissociable group, polyelectrolytes are subdivided into polyacids and polybases. Upon dissociation polyacids separate protons to give polyanions. Examples of polyacids are polyphosphoric acid, polyvinyl or polystyrene sulphuric acid, polyvinyl or polystyrene sulfonic acid, polyvinyl or polystyrene phosphonic acid and polyacrylic acid. Examples of the respective salts, which are also called polysalts, are polyphosphate, polysulphate, polysulfonate, polyphosphonate and polyacrylate. If the polyelectrolyte is a polycation, the nanoparticles preferably have an overall anionic charge.

Polybases contain groups which are capable of accepting protons, e.g. by reacting with acids to give salts. Examples of polybases are polyamines, such as polyethylene amine, polyvinyl amine and polyvinyl pyridine or poly(ammonium salts), such as poly (diallyl dimethylammonium chloride). Polybases form polycations by accepting protons. Preferably, polybases (i.e. polycations) are used as polyelectrolyte. If the polyelectrolyte is a polyanion, the nanoparticles preferably have an overall cationic charge.

The nanoparticles are preferably inorganic materials and may be selected from ceramic particles, e.g. oxidic ceramic particles, such as silicon dioxide, titanium dioxide, zirconium dioxide optionally doped with other metal oxides, magnetic particles such as iron oxide-containing particles such as Fe_3O_4, magneto-optical particles, nitridic ceramic particles, e.g. Si_3N_4, carbidic ceramic particles, metallic particles, e.g. gold, silver, palladium and sulfur or selene-containing particles such as cadmium sulfide, cadmium selenide etc. Especially preferred are oxidic ceramic particles such as silicon dioxide. It should be noted, however, that also organic or biological

nanoparticles are suitable for performing the present invention, e.g. macromolecules, such as polypeptides, proteins, nucleic acids, etc. Especially preferred biological nanoparticles are prot ins, e.g. immunologically reactive proteins such as antigens and antibodies, e.g. IgG, which may be deposited on the template particle alternating with polyelectrolyte molecules. The biological nanoparticles may be deposited as conjugates containing a labelling group, e.g. a fluorescent group such as fluorescein. The resulting particles are suitable for analytical, e.g. immunological, detection methods. Further, an immobilization of enzymes, single enzymes or a plurality of enzymes, e.g. members of an enzymatic cascade, in single layers or different layers is of special interest because of the ability to increase the catalysis efficiency. Substrates could readily diffuse through the film and react with the immobilized enzyme, thereby producing product.

For the preparation of the coated particles according to the present application preferably an aqueous dispersion of template particles of suitable size is provided. The aqueous dispersion may contain a salt, e.g. NaCl in a concentration which preferably ranges from 50 mmole/l to 1 mole/l. Alternating layers of oppositely charged components, i.e. polyelectrolyte molecules and nanoparticles, different types of nanoparticles or combinations thereof are then deposited on said template particles. The pH of the aqueous dispersion is adjusted in such a way that the molecules in each alternating layer, e.g. polyelectrolyte molecules and nanoparticles, each have opposite total charges. The thickness of the coating which is determined by the number of layers is preferably 2 to 1000 nm, with 2 to 40 and particularly 2 to 20, e.g. 3 to 10 coatings being applied. Suitably, each layer may be comprised of a single species of polyelectrolyte or nanoparticle or of a mixture comprising at least two polyelectrolyte or nanoparticle species. Further, for each layer different polyelectrolyte or nanoparticle species may be used.

After application of each layer the excessive molecules (e.g. polyelectrolyte or nanoparticle) which have not contributed to forming the layer are preferably separated off before the next layer is applied. Such separation can be done according to any known method, particularly centrifugation, filtration or/and dialysis.

In a preferred embodiment of the invention the template particles are at first coated with several layers of oppositely charged cationic and anionic polyelectrolytes before the alternating layers of nanoparticles and polyelectrolyte or the alternating nanoparticle layers are applied. The coating of template particles with several layers of oppositely charged polyelectrolytes is described in DE 198 12 083.4 to which express reference is made. Preferably, the template particles are coated with at least two and up to six layers of oppositely charged cationic and anionic polyelectrolytes, e.g. with three layers. The outermost polyelectrolyte layer is preferably oppositely charged with regard to the nanoparticle to be deposited.

The template particles can be of any shape whatsoever, e.g. they can be spherical or rod-shaped. They can have any regular or irregular structure including crystal structures. Furthermore, the template particles can also be composed of several small sub-particles.

The thickness of the shell walls around the template particles can be readily controlled by varying the number of deposition cycles, whilst the shell size and shape are predetermined by the dimensions of the template particle employed. The thickness of the shell can vary in a wide range, e.g. from 2 to 1000 nm, particularly from 5 to 250 nm.

Preferably, the template particles are at least partially disintegrated after the coating has been completed. They can be dissolved in appropriate solvents or thermally (e.g. by calcination to temperatures of at least 500°C) or - e.g.

if partially cross-linked melamine-formaldehyd template particles are used - by mild chemical methods, .g. in DMSO, or a change in the pH value. After dissolution of the t mplate particles hollow shells remain which ar composed of the nanoparticle material and optionally the polyelectrolyte material. The resulting hollow shells may be inorganic or organic shells or composite inorganic-organic or composite inorganic-inorganic shells depending on the method of core removal. For example, when thermal treatment (calcination) is used, all organic matter is removed, hence only inorganic shells are obtained. Exposure to solvent or low-pH solutions to remove the core results in composite hollow particles in which the core is removed but the polyelectrolyte assembled between the nanoparticle layers remains in the shell.

The hollow shells may be characterized by any known methods, e.g. by scanning and transmission electron microscopy and atomic force microscopy. Preferably, the shells are uniform layers of regular thickness and can find applications in numerous areas, such as medicine, pharmaceutics, catalysis, optics, magnetics, separation and sensing methods. In the hollow shells of the present invention active agents, e.g. inorganic or/and organic substances can be encapsulated. Examples of active agents are pharmaceutic agents so that the shells can be used as drug delivery systems in order to transport the active agents to the desired place in the organism. Further, also contrasting agents can be enclosed in the shells, e.g. to improve the quality of images obtained by ultrasonic examination. It is also possible that the hollow shells themselves are used as contrasting agents.

Further possible applications are fillers or pigments in paints, toners in printing or coatings. A still further field of application is the use as high surface area materials for catalysis , e.g. SiO_2 or TiO_2 shells, for solar energy applications, e.g. TiO_2 shells, wherein, if necessary, further active agents may be applied to the inner and/or outer side of the shells. Still

another application is the preparation of high-temperature ceramics, e.g. using zirconia nanoparticles or zirconia nanoparticles doped with other metal oxides. Moreover, the nanoparticles can also be used for the slow release of chemical substances, including pesticides, herbicides etc., as magnetic shells, e.g. for medical applications, or for separation and sensing procedures. Finally, the shells may be used as microreactors, e.g. to produce encapsulated colloidal particles, e.g. metal particles such as gold or silver particles, ceramic particles, magnetic particles or semiconductor particles.

Of particular importance for the use of shells is the permeability of the shell wall. The permeability of the shell wall can be influenced by the selection of the polyelectrolytes used for the shell, the wall thickness and the ambient conditions. It is thus possible to selectively determine and change the permeability properties.

The permeability properties can be further modified by pores in at least one of the layers. Such pores can be formed by the polyelectrolytes or nanoparticles themselves if suitably chosen. By incorporating selective transport systems such as carriers or channels into the polyelectrolyte shell the transversal transport properties of the shell can be adapted to the respective application. The pores or channels of the shell wall can be selectively opened and closed, respectively, by chemically modifying and/or changing the ambient conditions. A high salt concentration of the medium used for the deposition of the polyelectrolyte results in a low packing density and a high permeability of the shell wall. On the other hand, a high salt concentration of the medium used for the deposition of the nanoparticles (SiO_2) results in a high packing density of the silica particles. Thus, by adjusting the salt concentrations in the deposition medium, the permeability of the shell can be controlled, as desired. Further, the permeability properties of the shell may be modified by selecting the

conditions for decomposing the core, .g. by selecting the temperature and heating conditions in a calcination procedure.

A further aspect of the present invention is a coated particle having a core which is a template particle and a multilayer shell comprising alternating layers of (i) oppositely charged inorganic nanoparticles and polyelectrolytes or (ii) oppositely charged nanoparticles. Preferably, the average diameter of the coated particle is 15 μm or less, more preferably 100 nm to 10 μm.

Still a further aspect of the present invention is a hollow shell obtainable by disintegrating the template particles of the coated particle as described above. The hollow shell may be an inorganic structure or a composite organic-inorganic structure depending on the method used to remove the core.

Preferably, the shell contains an active agent which may be selected from pharmaceuticals, contrast agents, herbicides, pesticides, catalysts and pigments.

The shell may be used as a system for slow and/or targeted release of active substances such as pharmaceuticals, herbicides, pesticides etc. Further, the shell may be used for high surface area applications, e.g. as a carrier for catalysts or photovoltaic materials or as a catalyst itself.

It has been demonstrated that the method of the present invention when applied to produce composite nanoparticle-polymer or nanoparticle-nanoparticle multilayers on colloidal template cores, coupled with removal of the core, provides a successful pathway to fabricate novel hollow shells. Important advantages pertaining to this method are: (i) the thickness of the shell walls can be readily controlled by varying the number of deposition cycles; (ii) the shell size and shape are predetermined by the dimensions of the templating colloid employed; (iii) the method is generally applicable to

a wid variety of charged nanoparticles, thereby making possible the production of various inorganic, composite-inorganic (e.g. magnetic nanoparticle and SiO_2 or TiO_2) and composite inorganic-organic shell structures by the simple solution adsorption of charged particles in alteration with polymer; (iv) the method is a suitable alternative of those currently used (e.g. surface precipitation reactions) to produce hollow shells, with the added advantage of eliminating the necessity of more complex preparation procedures; and (v) it can be applied to systems for which the current methods are not suitable.

Further, the invention is illustrated by the following figures and examples:

Fig. 1 is a schematic illustration of the assembly of composite multilayers on colloid latices and the subsequent colloid and polyelectrolyte (optional) removal, resulting in hollow inorganic or composite shells. The first stage involves the sequential adsorption of oppositely charged polyelectrolytes, e.g. a Pr_3 coating [poly(diallyldimethylammonium chloride) (PDADMAC)/poly(styrene sulfonate), sodium salt (PSS)/ PDADMAC] (step 1) in order to produce a smooth and uniformly positively charged outer surface to facilitate the adsorption of negatively charged SiO_2 nanoparticles. Subsequent alternate adsorption of SiO_2 (step 2) and PDADMAC (step 3) results in SiO_2-PDADMAC multilayers being formed on the latices. The latices may be decomposed by calcination or exposure to low pH or solvent, whereby hollow inorganic or composite inorganic-organic shells are obtained.

Fig. 2 is a normalized light scattering intensity distribution of Pr_3-coated PS latices (squares) and PS latices coated with $Pr_3/(SiO_2/\ PDADMAC)_N$ wherein N = 1 (circles), N = 2

(triangl s) and N = 4 (diamonds). Multilayer growth is confirmed by the systematic shift in th SPLS intensity distributions.

Fig. 3 are transmission electron micrographs (TEM) of uncoated PS latices (a) and PS latices coated with $Pr_3/(SiO_2/PDADMAC)_N$ wherein N = 1 (b), N = 2 (c) and N = 4 (d). Regular growth of the SiO_2-PDADMAC multilayers is seen by an increase in diameter of the coated PS latices. The scale bar corresponds to all four TEM images shown.

Fig. 4 shows scanning electron micrographs (SEM) of PS latices coated with one SiO_2/PDADMAC multilayer (a) before and (b) after calcination. Uniform and smooth multilayer coatings are apparent in (a). The calcination process results in the PS latex core being removed (b): the thickness of the shell wall with one SiO_2 layer is not always sufficient to maintain the original sphericity of the PS latics (breakage of the hollow shells could also be caused by the vacuum in the SEM). Some intact shells were also observed with one SiO_2 layer.

Fig. 5 shows SEM micrographs of PS latices coated with three SiO_2/PDADMAC multilayers (a) before and (b) after calcination. Homogenous coatings are produced on the PS latices (a). Both complete, intact and broken shells are seen in (b): intact spheres are observed for the calcined sample with 3 SiO_2 layers; some of the shells are broken as a result of applying a force to them. The thickness of the silica wall is also significantly increased from that shown in Figure 4 (image a) as a result of successive SiO_2/PDADMAC depositions.

Fig. 6 is a higher magnification of Fig.5b.

Fig. 7 is an atomic force (AFM) image of a broken sphere wall.

Fig. 8 shows a TEM micrograph of hollow silica spheres produced by calcination of PS latices coated with two SiO_2/PDADMAC multilayers.

Fig. 9 shows a TEM micrograph of a cross section of silica hollow capsules obtained after calcination of PS latices coated with three SiO_2/PDADMAC multilayers. The calcined samples were embedded in a resin prior to imaging.

Fig. 10 shows an SEM image of hollow magnetite spheres. These hollow magnetic spheres are formed by depositing four layers of magnetite (Fe_3O_4) nanoparticles in alternation with PDADMAC on 640 nm PS latices and then calcining the sample.

Fig. 11 shows a TEM micrograph of a 3 μm melamine-formaldehyde particle coated with three SiO_2/PDADMAC layer pairs followed by exposure to a solution of pH = 1.

Fig. 12 shows the relationship of Zeta-potential and electrophoretic mobility as a function of polyelectrolyte layer number for FITC-BSA/PDADMAC (open squares) and IgG/PSS (filled squares) multilayers on polyelectrolyte-modified PS latex particles. FITC-BSA multilayers were formed on PDADMAC/PSS/PDADMAC-coated PS latex particles and IgG multilayers on $(PAH/PSS)_2$-coated particles. The odd layer numbers correspond to protein adsorption and the even layer numbers to polyelectrolyte deposition.

Fig. 13 shows Zeta-potential and electrophoretic mobility as a function of pH for a monomolecular IgG layer adsorbed on $(PAH/PSS)_2$-coated PS latex particles.

Fig. 14 shows fluorescence spectra of FITC-BSA in multilayer films of FITC-BSA/PDADMAC assembled onto PDADMAC/PSS/PDADMAC-precoated PS latex particles. The solid line corresponds to the spectrum of the multilayer film when FITC-BSA forms the outer layer, and the dashed line to that of the same film with PDADMAC deposited on top.

Fig. 15 shows normalised single particle light scattering (SLPS) intensity distributions of PDADMAC/PSS/PDADMAC-coated PS latex particles (a) and the same particles with one (b) and (c) three multilayers of FITC-BSA/PDADMAC. The final multilayer film structures on the colloids are: $[PDADMAC/PSS/PDADMAC/(FITC\text{-}BSA/PDADMAC)_N]$ wherein (a) N = 0, (b) N = 1, and (c) N = 3.

Fig. 16 shows FITC-BSA layer thickness (determined from SPLS) as function of protein layer number for FITC-BSA multilayers assembled on PDADMAC/PSS/PDADMAC-coated PS latex particles. The FITC-BSA multilayers were deposited in alternation with PDADMAC.

Fig. 17 shows the total film thickness (determined from SPLS) as a function of layer numbers for IgG/PSS multilayers assembled onto $(PAH/PSS)_2$-coated PS latex particles. The odd and even layer numbers correspond to protein and polyelectrolyte deposition, respectively.

Fig. 18 shows IgG layer thickness (determined from SPLS) as a function of protein layer number for IgG multilayers deposited in alternation with PSS on $(PAH/PSS)_2$-coated PS latex particles.

Fig. 19 shows TEM micrographs of PDADMAC/PSS/PDADMAC-coated PS latex particles (a and c) and the same particles additionally coated with $[(FITC\text{-}BSA/PDADMAC)_2/FITC\text{-}BSA]$ (b and d).

Fig. 20 shows TEM micrographs of IgG multilayers assembled onto $(PAH/PSS)_2$-coated PS latex particles. The final multilayer film structure on the particles is $[(PAH/PSS)_2/(IgG/PSS)_2/IgG]$. Image (b) is at a higher magnification than (a).

Example 1

Alternating SiO_2-poly(diallyldimethylammonium chloride) (PDADMAC) multilayers were prepared by first assembling a precursor three-layer PDADMAC and poly(styrene sulfonate) sodium salt (PSS) film (Pr_3) onto negatively charged 640 nm diameter polystyrene (PS) latices and then SiO_2-PDADMAC multilayers on the Pr_3-coated PS latices.

The Pr_3 film (PDADMAC/PSS/PDADMAC) was formed by the alternate adsorption of PDADMAC (Aldrich, $M_w < 200{,}000$) and PSS (Aldrich, M_w 70,000) from aqueous solutions: 0.5 mL of 1 mg mL^{-1} aqueous polyelectrolyte solution (containing 0.5 M NaCl) was added to the PS latices (10^{10} particles in 0.5 mL H_2O), 20 min allowed for adsorption, and excess polyelectrolyte removed by 4 repeated centrifugation (13500 x g)/wash/redispersion cycles. (SPLS measurements reveal that about 0.5% of the PS particles are lost at each wash/centrifugation/redispersion step). The Pr_3-coated PS latices exhibit a positive surface charge, as determined from electrophoretic mobility (EPM) measurements. Negatively charged

sulfate-stabilized PS latices were prepared as described in Furusawa et al, Kolloid-Z. u. Z. Polymere 1972, 250, 908.

SiO_2-PDADMAC multilayers on the PS latices were formed by adding 50 μL of an aqueous 40 wt% SiO_2 suspension (Ludox TM, DuPont) to the Pr_3-coated PS latices dispersed in 0.1 M NaCl (larger amounts of SiO_2 adsorb when the adsorbing solution contains NaCl), allowing 15 min for SiO_2 adsorption, removing excess SiO_2 by 4 repeated centrifugation (13500 x g)/wash/redispersion cycles, and subsequently depositing PDADMAC (1 mg mL^{-1}/0.5 M NaCl). The isoelectric point of the SiO_2 particles is 3, therefore SiO_2 is negatively charged under the conditions of adsorption (pH 5-6).

Electrophoretic mobility (EPM) measurements using a Malvern Zetasizer 4 show that the surface charge of the multilayer-coated particles alternates from negative to positive with each adsorption of SiO_2 and PDADMAC, respectively. This qualitatively demonstrates that the composite SiO_2-PDADMAC multilayers are formed by the step-wise adsorption of SiO_2 and PDADMAC.

The growth of the SiO_2-PDADMAC multilayers on the PS latices was first followed by the method of single particle light scattering (SPLS) (Lichtenfeld et al, Progr. Colloid Polym. Sci. 1997, 104, 148). Normalised SPLS intensity distributions for the Pr_3-coated PS latices and those coated with 1, 2 and 4 SiO_2-PDADMAC multilayers are shown in Figure 2. Deposition of the SiO_2-PDADMAC multilayers onto the PS latices is manifested as a shift (in the x-axis direction) in the SPLS intensity distributions, confirming multilayer growth. Using the SPLS technique, it is also possible to distinguish between singlets, doublets and triplets (Lichtenfeld et al, Progr. Colloid Polym. Sci. 1997, 104, 148): no aggregation of the multilayer-coated particles is observed, as no intensity peaks are observed at higher intensities.

Using the Raleigh-Debye-Gans theory (Kerker, The Scattering of Light and Other Electromagnetic Radiation: Academic Press: New York, London, 1969) and an estimated refractive index (n) of 1.40 for the adsorbed layer(s), the average thickness calculated for each SiO_2-PDADMAC layer pair for PS/Pr_3/(SiO_2/PDADMAC)$_N$ multilayers with N = 1-5 is 30 ± 6 nm. This value closely corresponds to the mean diameter of SiO_2 particles (26 ± 4 nm, determined from TEM), and suggests that on the average approximately a monolayer of SiO_2 is deposited with each SiO_2 adsorption. The layer thickness increases linearly with the number of SiO_2 or SiO_2-PDADMAC layers deposited onto the PS latices.

Direct observation of the multilayer growth process was provided by transmission electron microscopy (TEM) using a Philips CM12 microscope operating at 120 kV: representative TEM images of uncoated PS latices and Pr_3-modified PS latices coated with SiO_2-PDADMAC multilayers are displayed in Figure 3. The uncoated PS latices (a) exhibit a smooth surface. TEM images obtained for PS latices coated with Pr_3 are essentially identical to those of the uncoated PS latices: the thickness increase (ca. 4 nm, determined from SPLS experiments) is not discernible. The presence of SiO_2-PDADMAC multilayers on the PS latices results in both an increase in surface roughness and an increase in the diameter of the PS latices (b - d). The increase in surface roughness is due to adsorbed SiO_2. It was found that adsorption of PDADMAC onto an outermost layer of SiO_2 reduces the surface roughness of the multilayer. This finding is corroborated by preliminary scanning electron microscopy (SEM) measurements.

The increase in diameter with increasing SiO_2-PDADMAC multilayer number of the coated PS latices (relative to uncoated PS latices, a) is approximately 60 nm (b), 140 nm (c) and 250 nm (d), for 1, 2 and 4 SiO_2-PDADMAC multilayers, respectively. Evaluation of the TEM data for PS/Pr_3/(SiO_2/PDADMAC)$_N$ multilayers with N = 1 - 5 yields an average

diameter increment of 65 ± 5 nm, c rresponding to a layer thickness of ca. 32 nm, for the SiO_2-PDADMAC layer pair.

To remove the PS latex the solution was initially dried at room temperature and then placed into an oven under N_2 and heated to 500°C at a rate of 5 K/min. After 4h at this temperature the gas was changed to O_2 and the sample remained at 500°C for another 8h. The sample was then cooled to room temperature under O_2. The samples were then examined by SEM using a Zeiss DSM instrument operated at an accelerating voltage of 20 kV.

Figs.4 and 5 compare SiO_2/PDADMAC multilayer coated latex particles having different shell thicknesses before and after core removal. Fig.6 is a magnified picture of a hollow shell. It can be gathered from these results that intact hollow shells may be obtained which have sufficient permeability for the contents of the decomposed core to be expelled. Fig.7 shows an image of a broken sphere wall taken by atomic force microscopy (AFM), showing the wall is comprised of silica nanoparticles. The TEM micrograph of hollow silica spheres produced by calcination of PS latices coated with two SiO_2/PDADMAC in Fig. 8 shows the uniformity of the wall thickness. Upon careful examination two layers of silica nanoparticles can be observed. The uniformity of the shell wall thickness can also be seen from the TEM micrograph of resin-embedded silica hollow capsules as shown in Fig.9. The similar contrast both inside and outside the hollow spheres indicates that the spheres are permeable to the resin.

Example 2

According to the procedure described in Example 1 alternating Fe_3O_4-PDADMAC multilayers were deposited on 640 nm PS latex particles. After calcining the sample hollow magnetic spheres were formed. An SEM image of these spheres is shown in Fig.10.

Example 3

According to the procedure described in Example 1 alternating SiO_2-PDADMAC multilayers were deposited on 3 μm partially cross-linked melamine formaldehyde particles as described in DE 198 12 083.4. The melamine formaldehyde particles were dissolved by exposure to a solution of pH = 1. A TEM micrograph of a resulting hollow particle is shown in Fig.11. The diameter of the hollow composite sphere is significantly larger than the size of the MF colloid template due to drying of the sample on a solid substrate. The rough surface texture is due to the presence of SiO_2 nanoparticles embedded between polymer layers.

Example 4

Alternating protein-polyelectrolyte multilayers were deposited on colloid particles.

4.1 Materials

Fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) and sheep immunoglobulin G (IgG) were obtained from Sigma. Poly(allylamine hydrochloride)(PAH), M_w 8,000-11,000 or 50,000-65,000, poly(diallyldimethyl-ammonium chloride) (PDADMAC), M_w < 200,000, and poly(sodium 4-styrenesulfonate) (PSS), M_w 70,000, were purchased from Aldrich. All proteins and polyelectrolytes were used as received, except for the 70,000 M_w PSS which was dialyzed against water (M_w cut-off 14,000) and lyophilized before use. The negatively charged sulfate-stabilized polystyrene (PS) latex particles (diameter 640 nm) were prepared as described in Example 1.

4.2 Assembly of protein multilayers onto colloids

Precursor polyelectrolyte multilayer films consisting of PAH and PSS or PDADMAC and PSS were first deposited in order to provide a uniformily charged surfac and to facilitate subsequent prot in adsorption. The precursor films were formed as described in Example 1.

Protein multilayers were fabricated by exposure of the polyelectrolyte-coated PS latex particles to protein solution under conditions where the protein and particles bear opposite charges, followed by alternate adsorptions of polyelectrolyte and protein. FITC-BSA was deposited onto (PDADMAC/PSS/PDADMAC)-precoated PS latex particles, and IgG onto $(PAH/PSS)_2$-precoated particles. The particles have a positive surface charge when PAH or PDADMAC form the outermost layers, and a negative charge when PSS is the outermost layer. FITC-BSA multilayers were formed by the alternate adsorption of FITC-BSA (0.5 mg/ml in PBS buffer at pH 7.0, 30 min adsorption, or 1 mg/ml in water at pH $\approx$ 5.6, 20 min adsorption) and PDADMAC (1 mg ml/0.5 M NaCl, 20 min) onto the coated PS latex particles (5 x 10^9 particles). IgG multilayers were prepared by successive adsorptions of IgG (1 mg/ml in 0.05 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 6.0 pH adjusted using NaOH, 45 min adsorption) and PSS (M_w 8,000 - 11,000, 1 mg/ml/0.5 M NaCl, 20 min) layers onto the coated PS latex particles (6 x 10^9 particles). (In all cases the concentration of the protein is approximately ten times that required for saturation adsorption of the particle surface). After each deposition of protein or polyelectrolyte layer, the samples were centrifuged at ca. 5000 x g for 10 min, the supernatant removed, and at least three water washings performed.

4.3 Electrophoretic mobility (EPM) measurements

Electrophoretic mobilities of the bare and coated PS latex particles were measured using a Malvern Zetasizer 4 as described in Example 1. All Zeta-

potential measurements were performed on coated PS latex particles re-dispersed in air-equilibrated pure water (pH ≈ 5.6).

4.4 Single particle light scattering (SPLS) experiments

Details of the SPLS experimental system and measurement principle were as described in Example 1. Briefly, the dispersion (of either uncoated or coated PS latex particles) is passed through a capillary with a 0.1 mm diameter orifice at the end. Hydrodynamic focussing is applied so that the dispersion stream is directed through a laser beam which is focussed to allow only a single particle or aggregate in focus at a particular time. This requires particle concentrations of less than 3×10^8 particles ml^{-1}. The light scattered by the particles moving through the laser focus is recorded in the angular region of 5 - 10° in the forward direction. The intensity distributions, obtained with a resolution of 0.5%, are collected by a multi-channel analyser and then stored on a PC.

4.5 Transmission electron microscopy (TEM)

TEM measurements were performed on a Philips CM12 microscope operating at 120 kV. Samples for TEM were prepared by deposition of aqueous solutions of the coated PS latex particles upon a carbon-coated copper grid. The mixtures were allowed to air dry for one minute and the extra solution was then blotted-off.

4.6 Steady-state fluorescence measurements

Fluorescence spectra were recorded using a Spex Fluorolog 1680 spectrometer with excitation and emission bandwidths set at 1.0 nm. Typically, ca. 50 - 100 μl of the protein multilayer-coated PS latex suspension (ca. 10^9 particles ml^{-1}) was pipetted into 3 ml of water in a

fluorimeter cell and the dispersion agitated for 0.5 min. The fluorescence spectrum of this dispersion was then recorded.

4.7 Results

The assembly of protein multilayers was first followed by EPM measurements. Prior to the formation of protein multilayers, a precursor three or four layer polyelectrolyte multilayer film was deposited onto the PS latex particles. The precursor film not only provides a uniformly charged surface, which facilitates subsequent protein adsorption, but it also allows the surface charge to be altered (depending on whether the polycation or polyanion forms the outermost layer) so that the protein can be deposited under conditions where it is oppositely charged to the adsorbing surface. A three layer (PDADMAC/PSS/PDADMAC) film was assembled onto the negatively charged PS latices prior to deposition of FITC-BSA, whilst a four layer $(PDADMAC/PSS)_2$ film was deposited onto the particles before IgG adsorption. The negatively charged (uncoated) PS latex particles have a Zeta-potential of about -65 mV in water. The Zeta-potential of the PDADMAC/PSS/PDADMAC-coated particles is ca. +50 mV, in accordance with the outermost layer being a polycation. For the $(PDADMAC/PSS)_2$-coated PS latex particles, the Zeta-potential is ca. -40 mV, consistent with the outermost layer being the polyanion PSS. Figure 12 shows the Zeta-potential as a function of layer number for the polyelectrolyte-modified PS latex coated with FITC-BSA/PDADMAC or IgG/PSS multilayers. The alternate assembly of FITC-BSA and PDADMAC causes a reversal in sign of the Zeta-potential with each deposition up to 10 layers. When FITC-BSA forms the outermost layer, the Zeta-potential of the coated particles is slightly negative (-10 to -20 mV). Subsequent adsorptions of PDADMAC and FITC-BSA produce positive and negative Zeta-potentials, respectively. For the IgG/PSS multilayer system, Zeta-potential values close to 0 or slightly negative are observed when IgG is the outermost layer. Thus, a

reversal of surface charge is not required in the build-up of monoparticle/monoelectrolyte multilayers, e.g. IgG/PSS.

EPM measurements where the Zeta-potential was recorded as a function of pH were conducted on $(PAH/PSS)_2$-modified PS latex particles coated with *one* IgG layer (Figure 13). The results show an isoelectric point of approximately 5.5.

Further evidence for the growth of FITC-BSA/PDADMAC multilayers was provided by fluorescent measurements. The fluorescence spectra for FITC-BSA/PDADMAC multilayers, for the cases where FITC-BSA or PDADMAC form the outermost layer, are shown in Figure 14. The emission maximum occurs at 515 nm when the outer layer is FITC-BSA. When PDADMAC is the outer layer, this maximum red-shifts by about 6 - 7 nm to 521 - 522 nm. Reproducible, oscillating maxima were observed for each deposition of FITC-BSA and PDADMAC.

In order to obtain quantitative evidence of step-wise protein multilayer growth, the technique of SPLS was employed. SPLS is a sensitive optical technique which enables determination of the thickness of layers assembled onto colloids, as well as the state and degree of the coated colloids with respect to aggregation. By passing a dispersion of the coated particles through a capillary and hydrodynamically focusing the dispersion, the light scattered from one particle at a given moment in time is recorded. Repeating this process allows a histogram of particle number versus scattering intensity to be obtained. Figure 15 shows the normalized SPLS intensity distributions for (PDADMAC/PSS/PDADMAC)-modified PS latex particles (a), and the same particles coated with one (b) and three (c) FITC-BSA/PDADMAC multilayers. There is a systematic shift in the SPLS intensity distribution (in the x-axis direction) with increasing multilayer layer number, confirming the growth of FITC-BSA/PDADMAC multilayers on PS latex particles. Similar SPLS intensity distributions were obtained for the

IgG/PSS multilayers on PS particles. The peaks seen in the SPLS curves shown in Figure 15 correspond to singlets, i.e. unaggregated protein multilayer-coated particl s. Analysis of the data revealed that th coated particles exist predominantly as singlets, with less than 20% of the particles as doublets (an aggregate of two particles). The fraction of doublets was considerably reduced (< 5%) when the polyelectrolyte was outermost layer. This indicates that adsorption of the polyelectrolyte separates some of the weakly and reversibly flocculated protein multilayer-coated particles.

By using the Rayleigh-Debye-Gans theory, and refractive indices (n) of 1.43 and 1.47 for the protein and polyelectrolyte layers, respectively, the average thickness of the protein/polyelectrolyte multilayers on the PS latex particles (d) can be determined. For the FITC-BSA multilayers, the layer thickness increases linearly with the number of protein layers deposited (Figure 16). The calculated average layer thickness increment for the FITC-BSA layers is 3.3 ± 1.1 nm when FITC-BSA is adsorbed from pure water, and 5.8 ± 2.5 nm when adsorbed from PBS. The difference in thickness is attributed to the different conditions under which the protein was deposited. These data clearly show that BSA multilayers can be grown by the step-wise adsorption of protein and PDADMAC onto PS latex particles.

Figure 17 shows the layer thickness of IgG/PSS multilayers assembled on $(PAH/PSS)_2$-coated PS latex particles as a function of layer number. Regular, step-wise multilayer growth is observed. The IgG multilayer film growth is linear after the first deposition step, as shown in Figure 18. The thickness of the first IgG layer deposited is approximately 11 nm. The IgG average thickness increment after the first deposition cycle (of IgG and PSS) is 37 ± 7 nm.

Direct visualization of the protein multilayer growth process is provided by TEM. Figure 19 shows the TEM micrographs of PDADMAC/PSS/PDADMAC-modified PS latex particles (a and c) and the same particles coated with

(FITC-BSA/PDADMAC)$_2$/FITC-BSA multilayers (b and d). The polyelectrolyte-coated PS latex particl s closely resemble uncoated PS latices in appearance: they exhibit a smooth surfac . The thickness of the polyelectrolyte coating (PDADMAC/PSS/PDADMAC) is approximately 3 - 4 nm (from SPLS experiments). The presence of FITC-BSA multilayers on the PS latices produces both an increase in surface roughness and an increase in the diameter of the polyelectrolyte-coated PS latices (b and d). The increase in surface roughness is most notable at higher magnification (compare images a and d). The diameter increase of the particles with (FITC-BSA/PDADMAC)$_2$/FITC-BSA layers is approximately 20 nm, corresponding to a layer thickness increase of about 10 nm. This value is in close agreement with the SPLS thickness for the same multilayer (11 nm). The TEM images for FITC-BSA multilayer-coated PS latex particles confirm that a uniform coating of the particles is obtained with deposition of FITC-BSA multilayers (Figure 19b and 19d).

The TEM micrographs for the IgG/PSS multilayer-coated PS latex particles (Figure 20) clearly demonstrate a regular layering of the particle surface by the protein multilayers. The diameter of the IgG multilayer particles is significantly larger than those onto which the multilayers were formed. The deposition of one, three and five IgG layers resulted in an increase in diameter of approximately 16, 164 and 296 nm, respectively. These values correspond to layer thickness increases of 8, 82 and 148 nm, and are in excellent agreement with those calculated from SPLS measurements (11, 90 and 160 nm for 1, 3 and 5 IgG layers, respectively).

Example 5

PS latex particles (640 nm in diameter) were used as substrate for the adsorption of polyelectrolyte and enzyme layers. The layering procedure started with the adsorption of four alternating PAH (Mw 50 - 65 kD) and PSS (Mw about 70 kD) layers onto the PS latex resulting in negatively

charged particles with PSS at the outermost coating. Polyelectrolytes were allowed to adsorb within 20 min. Each layering step was follow d by four washing cyles with deionized water. After every layering and washing st p the mixtures were centrifuged at 7000 rpm for 8 min and the supernatants were removed. Onto the last PSS layer β-glucosidase from Caldocellum saccharolyticum obtained from Sigma (EC 3.2.1.21) was adsorbed in one hour. The enzyme was used as 1.4 mg/ml solution in 0.1 M acetate buffer pH = 4.8. The described procedure was repeated until four enzyme layers each separated by one PSS layer were assembled on the particles. Samples were taken after each layering step yielding samples with 1 to 4 enzyme layers having either enzyme or PSS as the outermost coating. These samples were used as catalysts in enzymatic glucosidations.

Glucosidations were carried out in 2 ml tubes which were shaken (200 min^{-1}) at 40°C. Enzyme modified latex particles were suspended in 100 μl phosphate buffer pH = 6.8 solution. In a typical experiment 413 mg (2.22 mmoles) n-dodecanol (Fluka), 600 μl acetonitrile (Merck), and 100 mg (0.55 nmoles) anhydrous glucose (Merck) were added. The mixtures were shaken thoroughly to homogenize and then kept at 40°C for 72 hours with shaking. The mixtures were then centrifuged at 5000 rpm for 10 min. The supernatants were removed and the remainders were washed with 500 μl acetonitrile. From the combined organic phases of each sample the solvent was distilled off. The concentrated crude products were subjected to a preparative chromatographic separation to remove excess dodecanol.

Preparative liquid chromatography was done with a Büchi-680 HPLC equipment consisting of chromatography pump, gradient former, fraction collector, and a Knauer polar monitor detector. The column was a Kronlab bio-cart (20 mm x 120 mm) packed with 10 g of 15 μm spherical silica. By collecting 10 ml fractions with an ethyl acetate/methanol gradient program (0 to 100% methanol, 34 min) all excess dodecanol could be separated from the crude product. The remaining fractions were combined,

concentrated and then fixed to a volume of 1 ml by adding a water/propane-2-ol (1:33 v/v) mixture. To determine the amount of formed dodecyl glucoside aliquots of these stock solutions were analyzed by HPLC on a JASCO chromatograph equipped with a light scattering detector and silica column. For the quantitative determination of dodecyl glucoside calibration curves were constructed.

In the case of particles having enzyme or PSS as outermost layers dodecyl glucoside could be detected. Thus, the enzyme is not inactivated by the coating procedure. In the case of particles having PSS as the outermost layer an increasing yield of dodecyl glucoside with increasing numbers of deposited enzyme layers on the particles was observed. This shows that also layers underneath take part in the catalytic process.

Claims

1. A process for preparing coated particl s comprising the steps:
   (a) providing template particles and
   (b) coating said template particles with a multilayer comprising (i) alternating layers of oppositely charged nanoparticles and polyelectrolytes and/or (ii) alternating layers of oppositely charged nanoparticles.

2. The process of claim 1 wherein said template particles have an average diameter of 10 μm or less.

3. The process of any one of claims 1 or 2 wherein said template particles are selected from organic particles, inorganic particles, or any combinations thereof.

4. The process of any of claims 1 to 3 wherein said template particles are selected from organic polymer latices and partially cross-linked melamine-formaldehyde particles.

5. The process of any one of claims 1 to 4 wherein the polyelectrolyte is a linear molecule.

6. The process of any one of claims 1 to 5 wherein the polyelectrolyte is a polycation and the nanoparticle has an overall anionic charge.

7. The process of any of claims 1 to 5 wherein the polyelectrolyte is a polyanion and the nanoparticle has an overall cationic charge.

8. The process of any one of claims 1 to 7 wherein said nanoparticles have an average diameter of from 1 to 100 nm.

9. Th process of any one of claims 1 to 8 wherein said nanoparticles are inorganic particles.

10. The process of claim 9 wherein said nanoparticles are selected from ceramic and metal particles.

11. The process of claim 10 wherein said nanoparticles are silicon dioxide particles.

12. The process of any one of claims 1 to 8 wherein said nanoparticles are biomolecules.

13. The process of claim 12 wherein said biomolecules are polypeptides.

14. The process of any one of the preceding claims further comprising the step:
(c) at least partially disintegrating the template particles.

15. The process of claim 14 wherein said disintegration is carried out by thermal treatment, chemical treatment or pH adjustment.

16. Coated particle having a core which is a template particle and a multilayer shell comprising alternating layers of (i) oppositely charged nanoparticles and polyelectrolytes or (ii) oppositely charged nanoparticles.

17. The particle of claim 16 having an average diameter 15 μm or less.

18. Hollow shell obtainable by disintegrating the template particle of the coated particle of claim 16 or 17.

19. The hollow shell according to claim 16 which is an inorganic structur .

20. The hollow shell according to claim 16 which is an organic structure.

21. The hollow shell according to claim 16 which is a composite organic-inorganic structure.

22. The hollow shell according to claim 16 which is a composite inorganic-inorganic structure.

23. The shell of any one of claims 18 to 22 containing an active agent.

24. The shell of claim 23 wherein said active agent is selected from pharmaceuticals, contrasting agents, herbicides, pesticides, catalysts, and pigments.

25. Use of the shell according to any one of claims 18 to 24 as a system for slow and/or targeted release of active substances.

26. Use of the shell according to any one of claims 18 to 24 for high surface area applications.

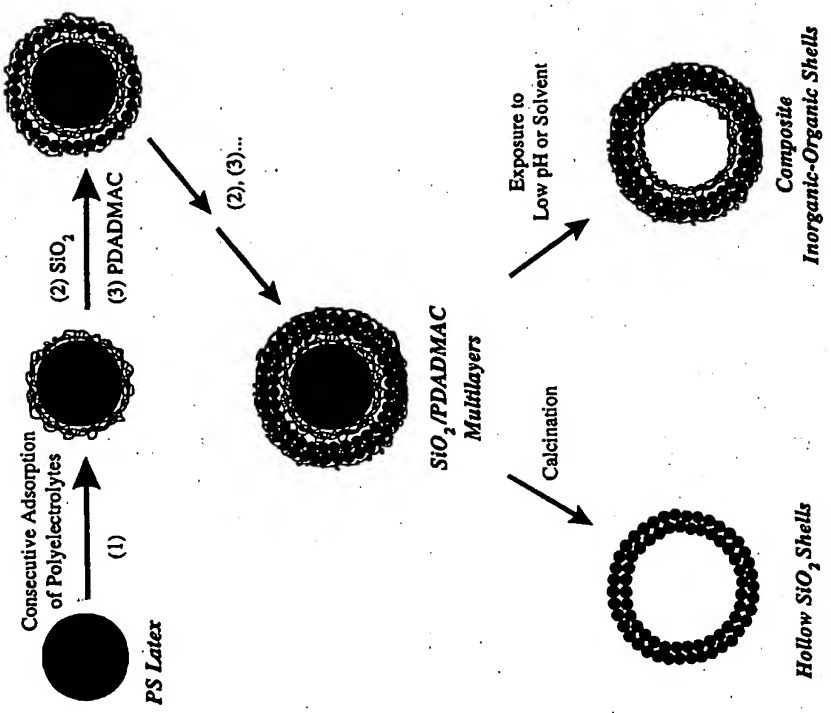

Consecutive Adsorption of Polyelectrolytes
(1)
PS Latex
(2) SiO2
(3) PDADMAC
(2), (3)...
SiO2/PDADMAC Multilayers
Exposure to Low pH or Solvent
Composite Inorganic-Organic Shells
Calcination
Hollow SiO2 Shells

Figure 1

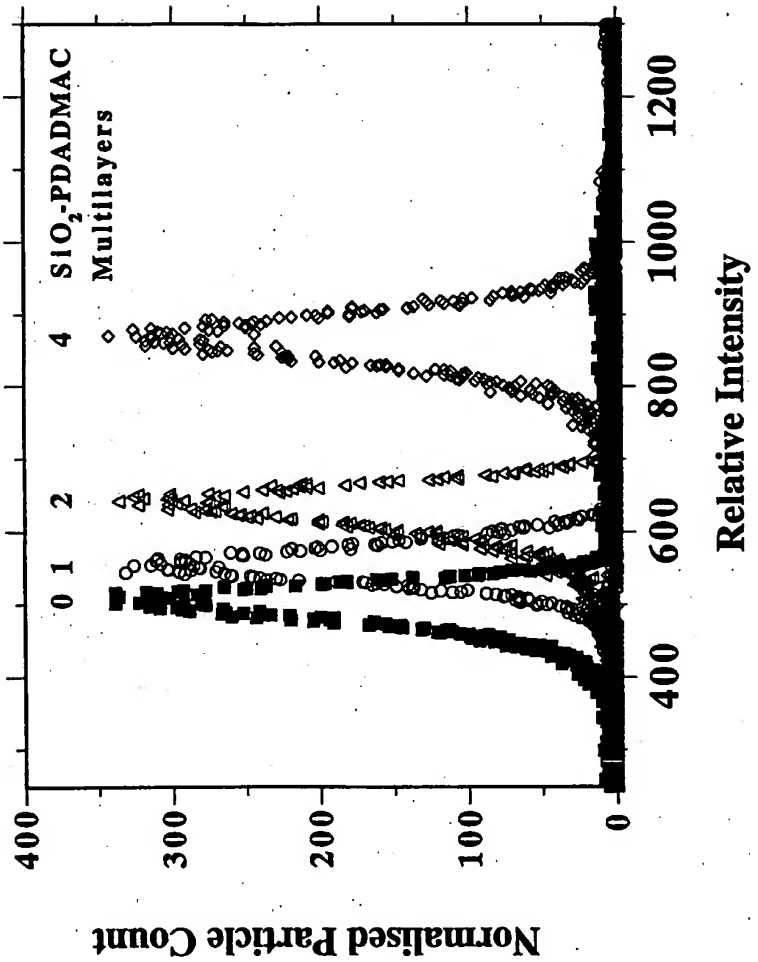

SiO2-PDADMAC
Multilayers
0 1 2 4
Normalised Particle Count
Relative Intensity
0
100
200
300
400
400
600
800
1000
1200

Figure 2

Figure 3

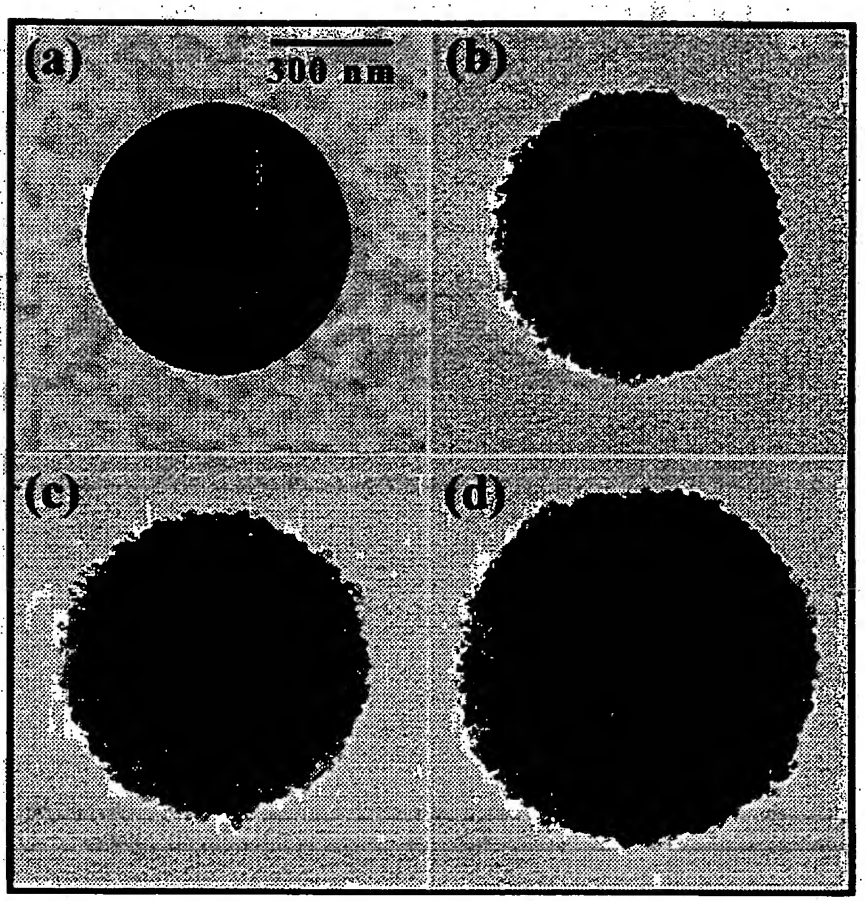


Figure 4

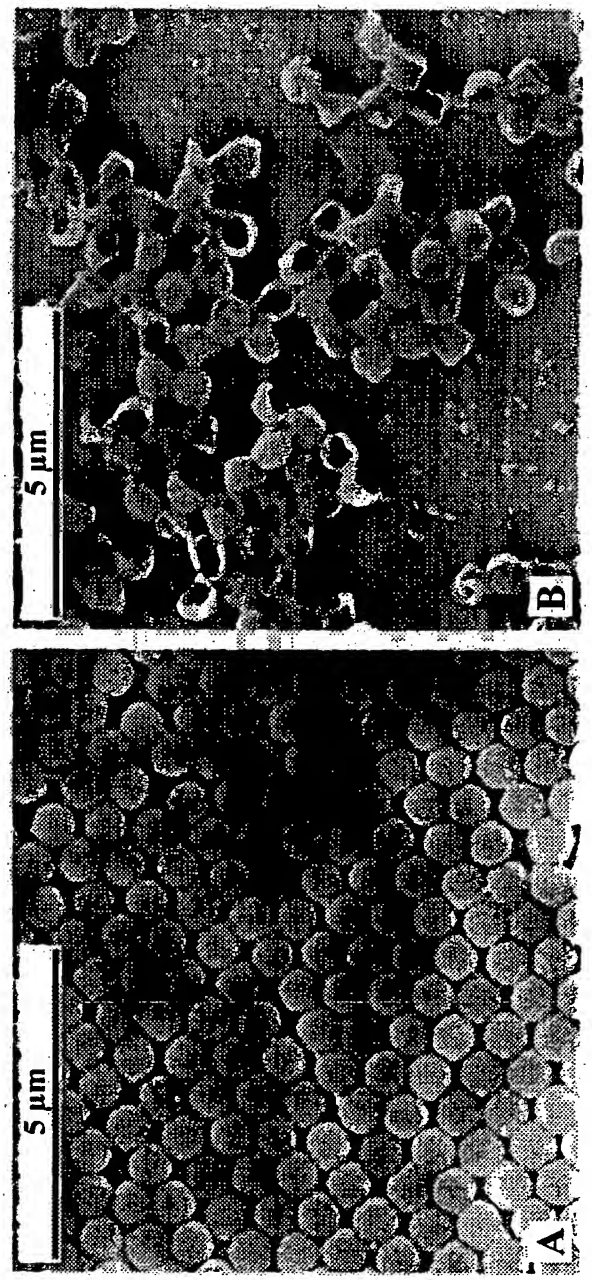
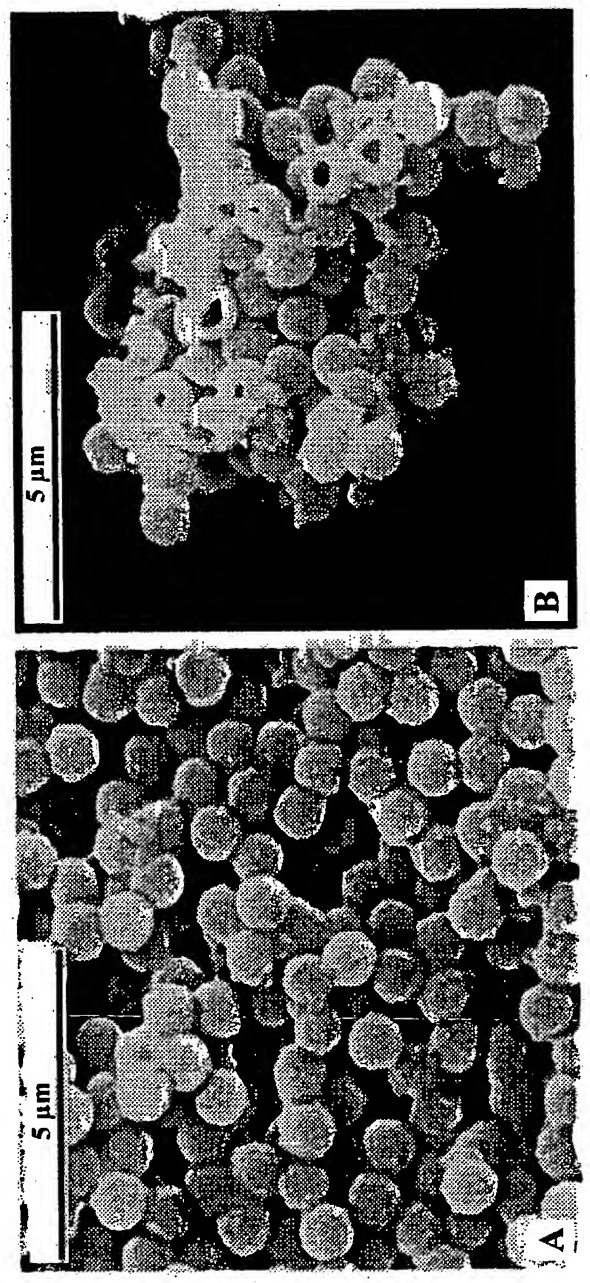


Figure 5



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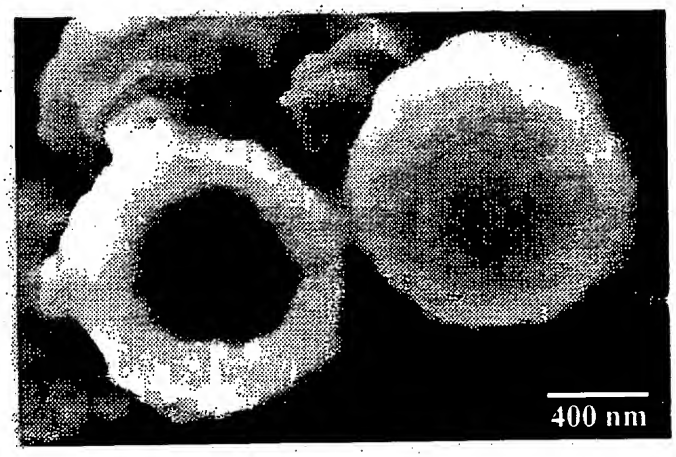


Figure 7

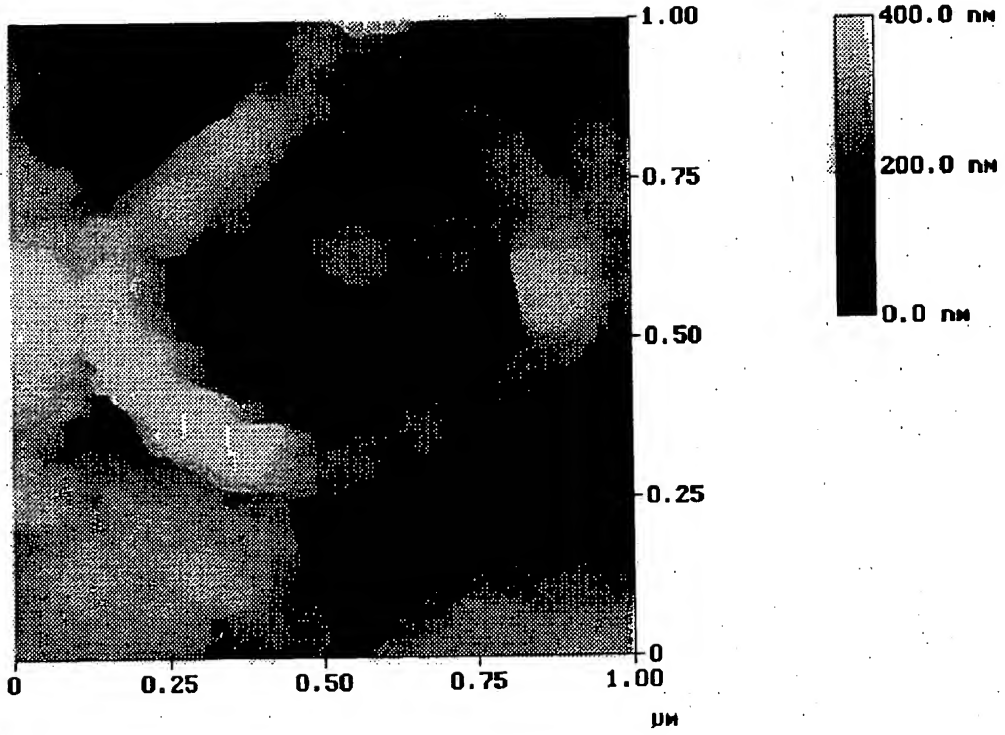


Figure 8

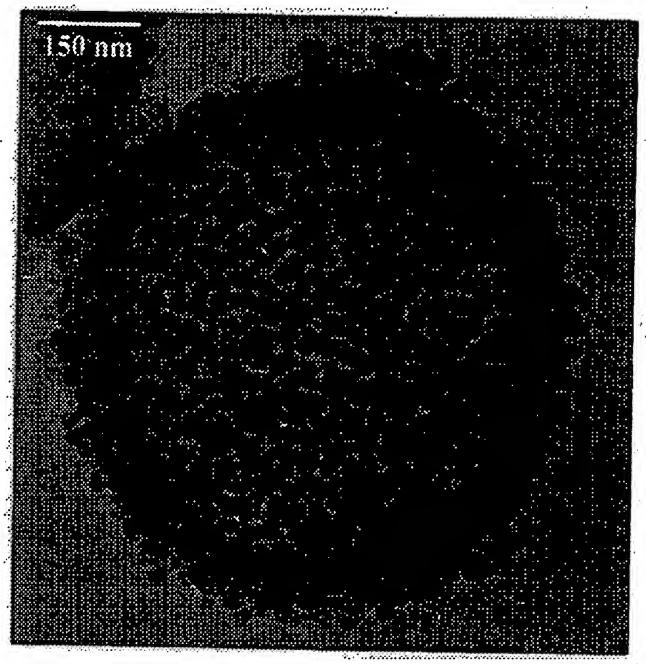


Figure 9

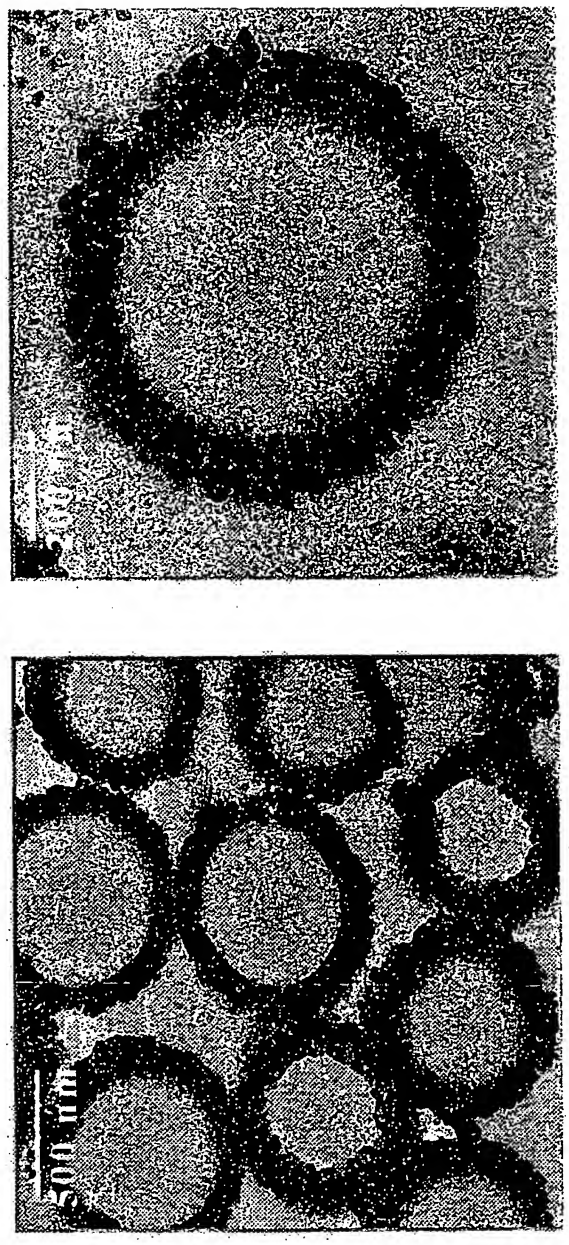


Figure 10

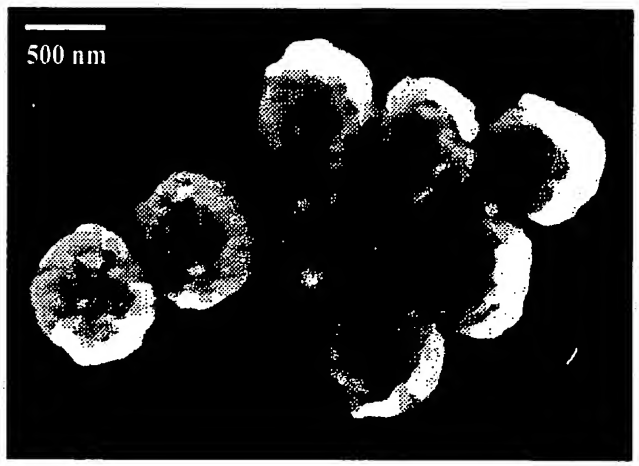


Figure 11

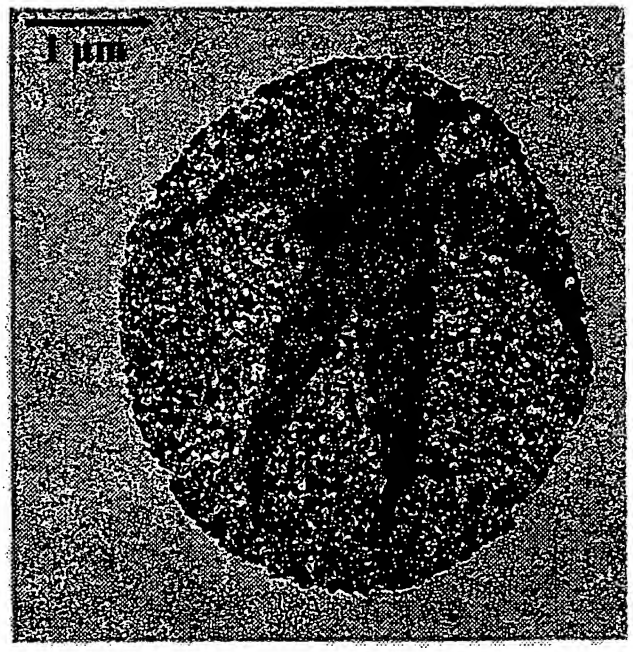


Figure 12

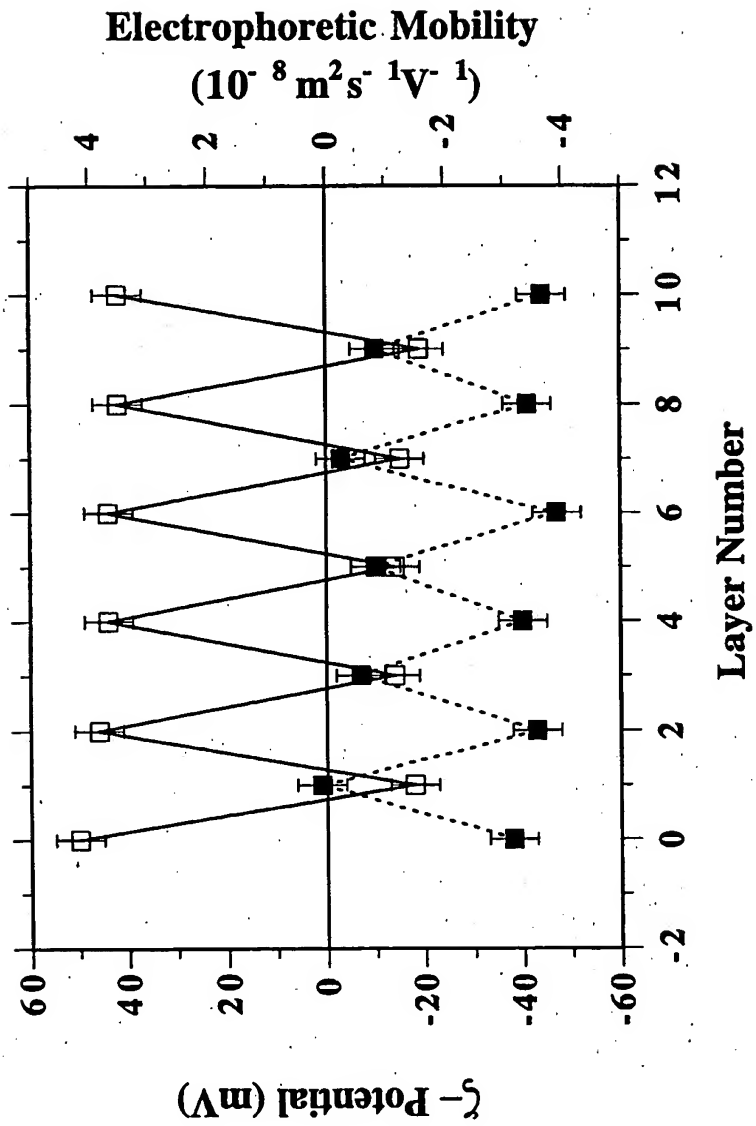
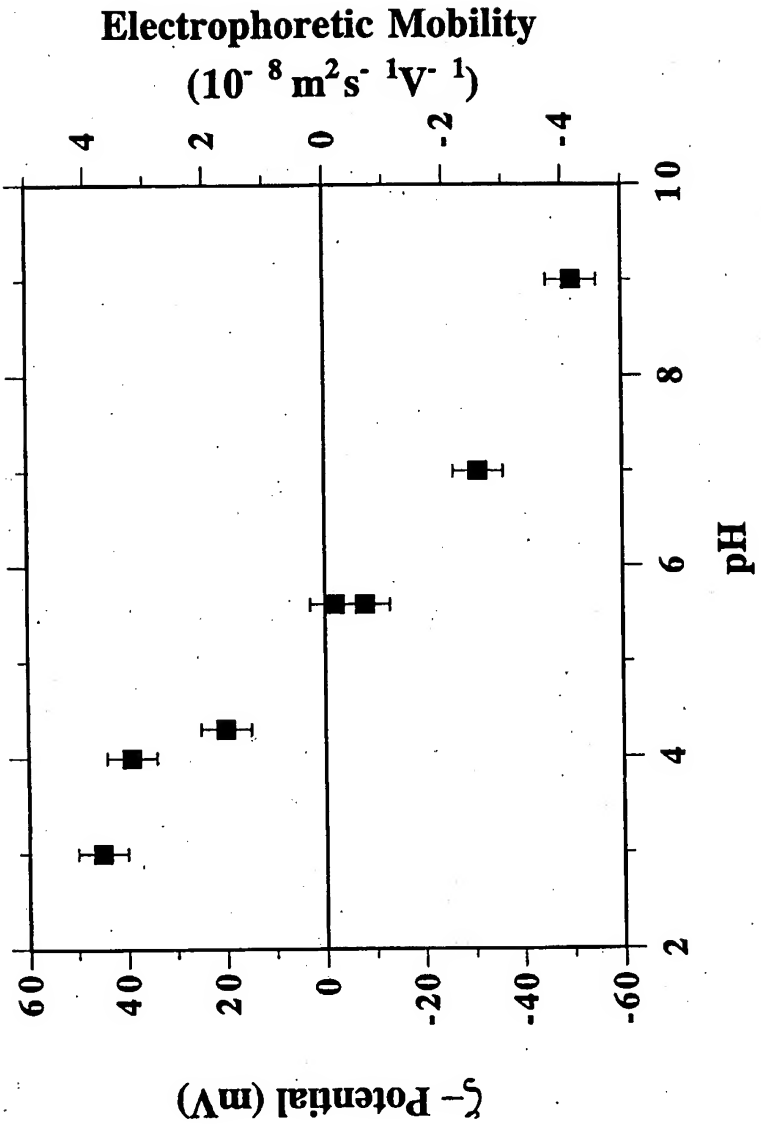

Electrophoretic Mobility
(10⁻⁸ m²s⁻¹V⁻¹)
4
2
0
-2
-4
12
10
8
6
4
2
0
-2
Layer Number
60
40
20
0
-20
-40
-60
ζ– Potential (mV)

Figure 13


Electrophoretic Mobility
(10- 8 m2s- 1V- 1)
4
2
0
-2
-4
10
8
6
4
2
pH
60
40
20
0
-20
-40
-60
ζ- Potential (mV)

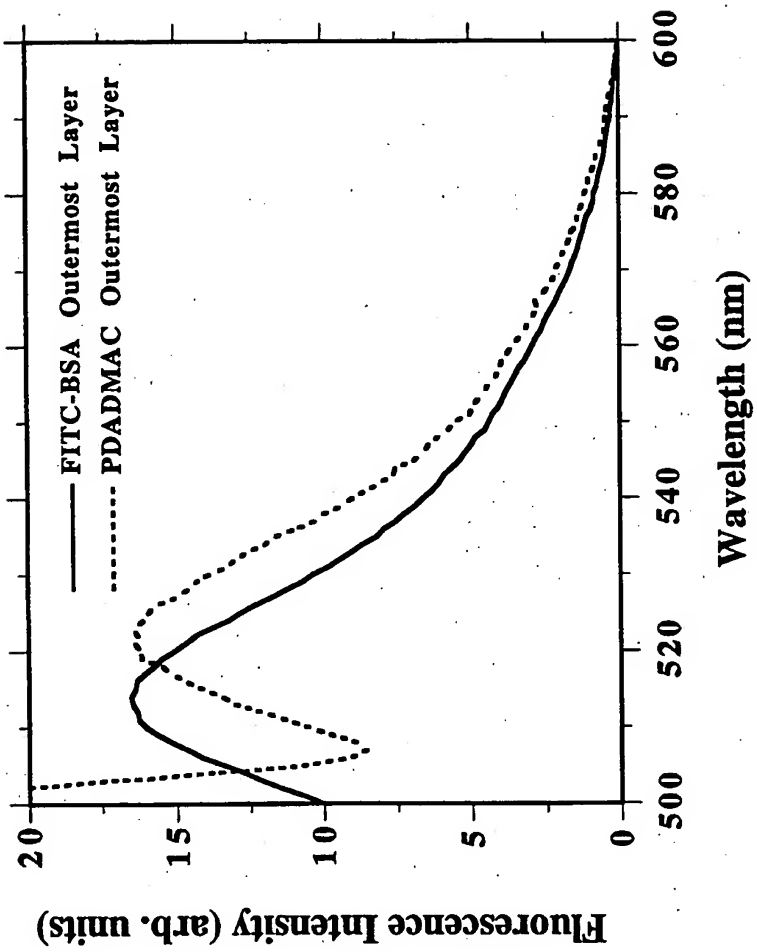

FITC-BSA Outermost Layer
PDADMAC Outermost Layer
Fluorescence Intensity (arb. units)
Wavelength (nm)
0
5
10
15
20
500
520
540
560
580
600

Figure 14

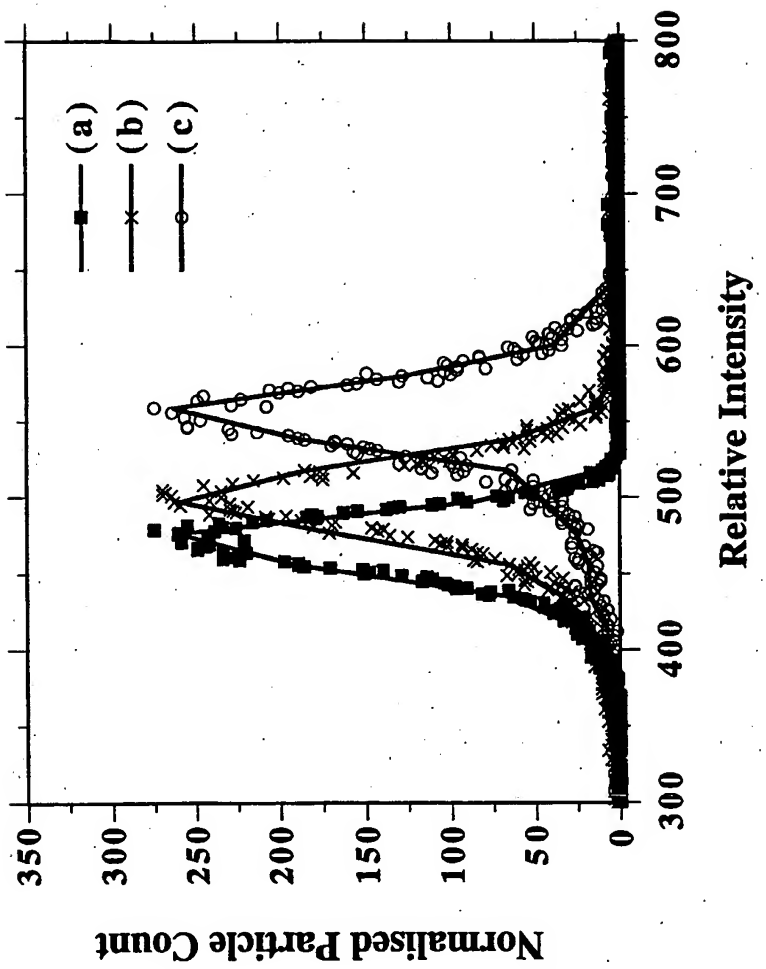

Normalised Particle Count
0
50
100
150
200
250
300
350
Relative Intensity
300
400
500
600
700
800
(a)
(b)
(c)

Figure 15

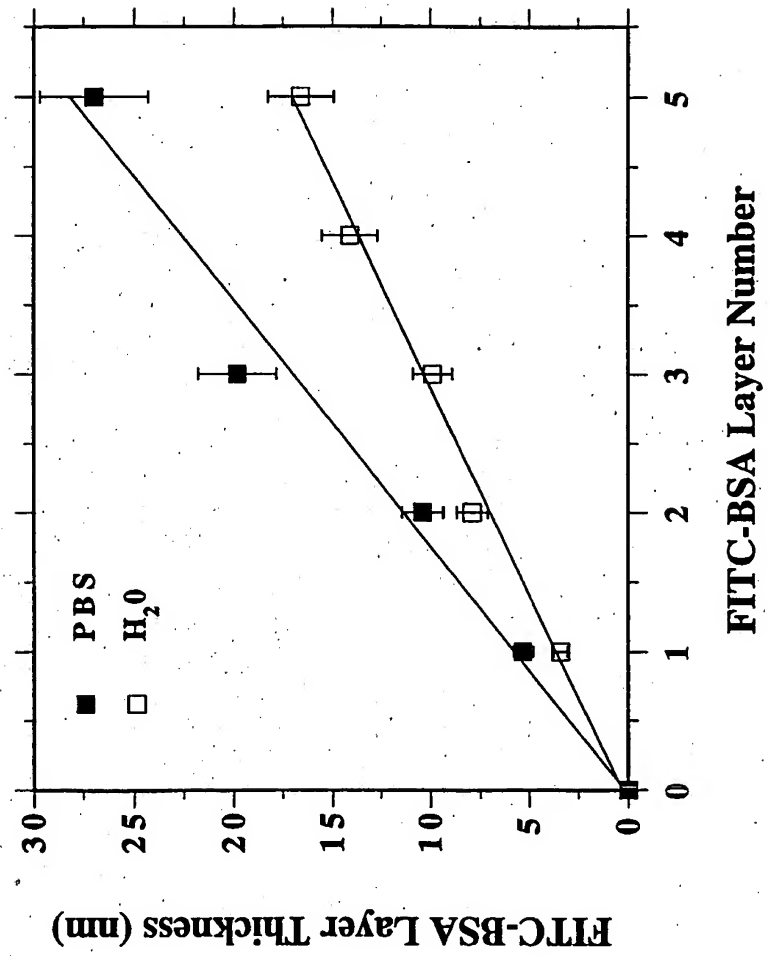

FITC-BSA Layer Thickness (nm)
FITC-BSA Layer Number
PBS
H_2O
0
5
10
15
20
25
30
0
1
2
3
4
5

Figure 16

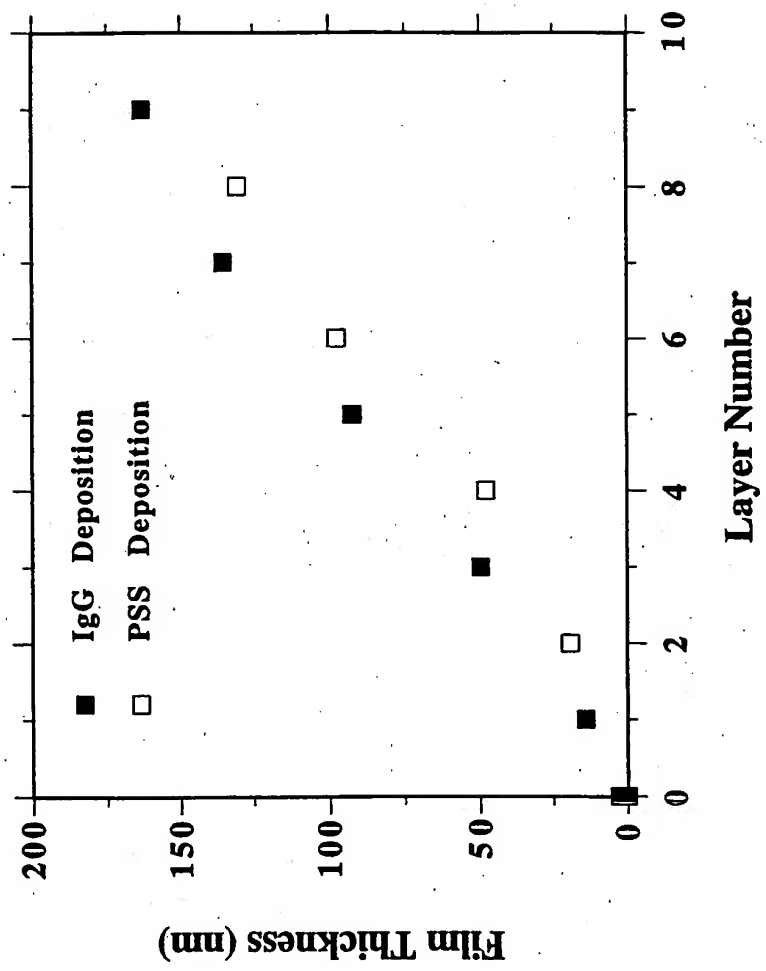

Film Thickness (nm)
200
150
100
50
0
0
2
4
6
8
10
Layer Number
IgG Deposition
PSS Deposition

Figure 17

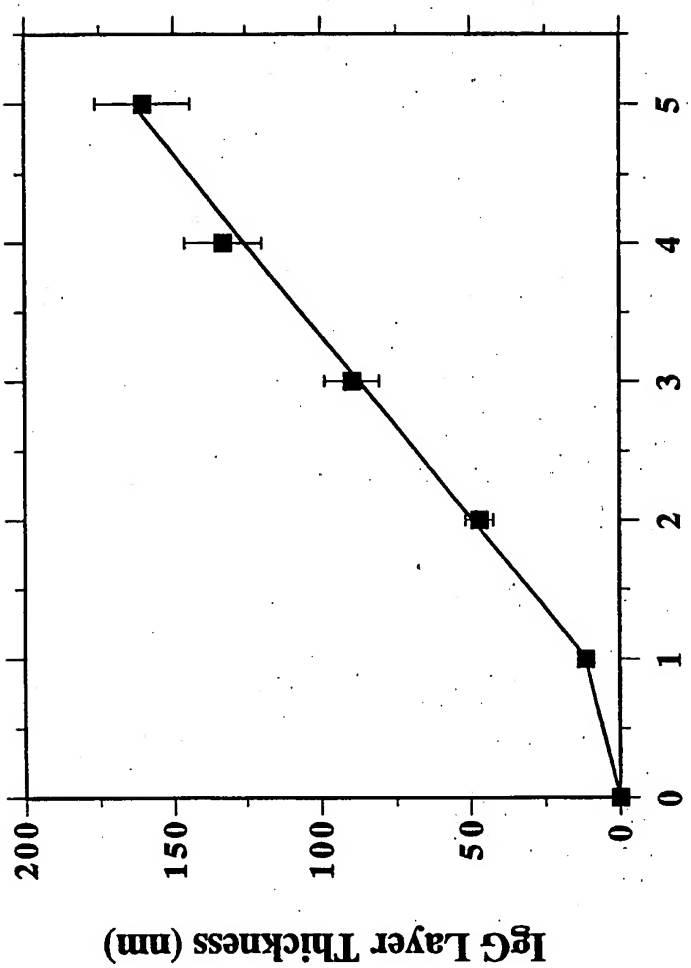

IgG Layer Thickness (nm)
200
150
100
50
0
0
1
2
3
4
5
IgG Layer Number

Figure 18

Figure 19

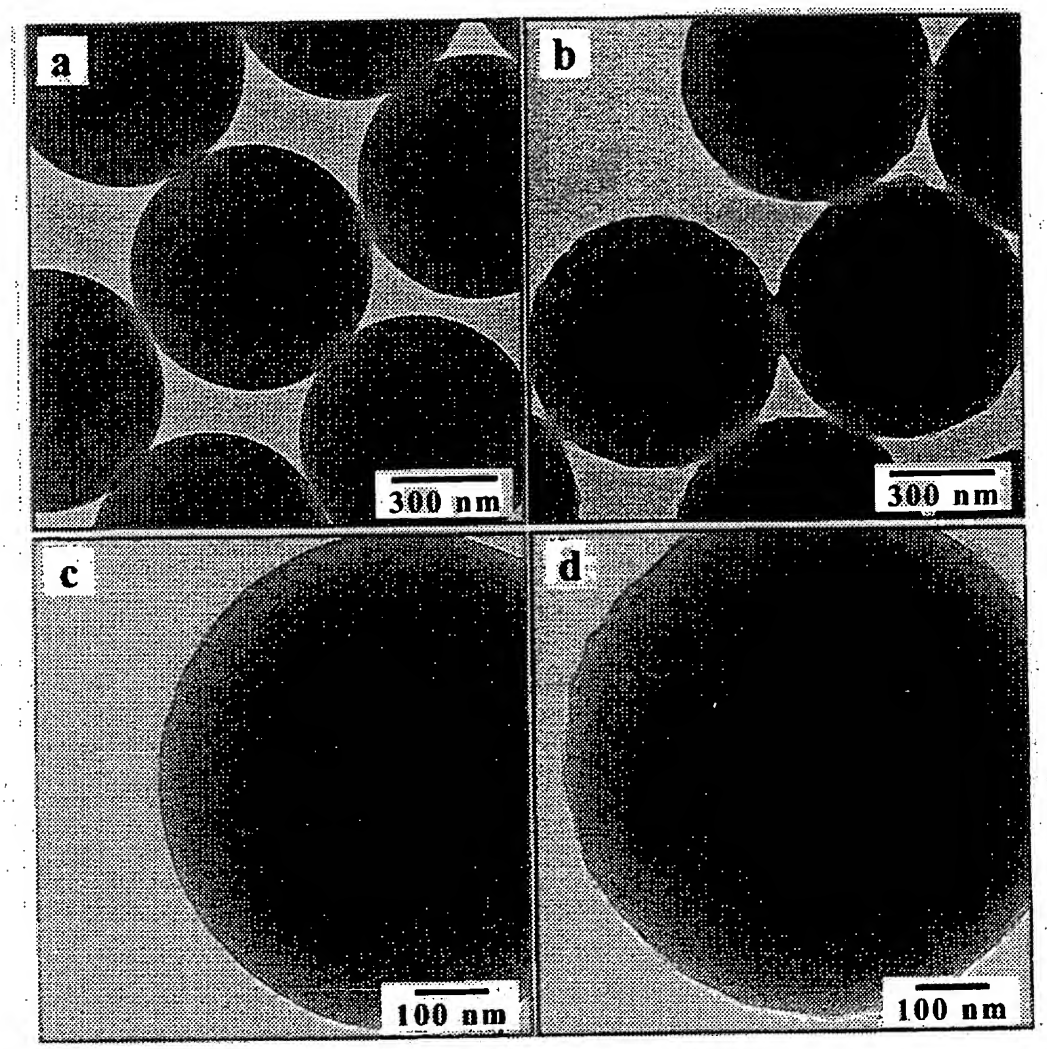
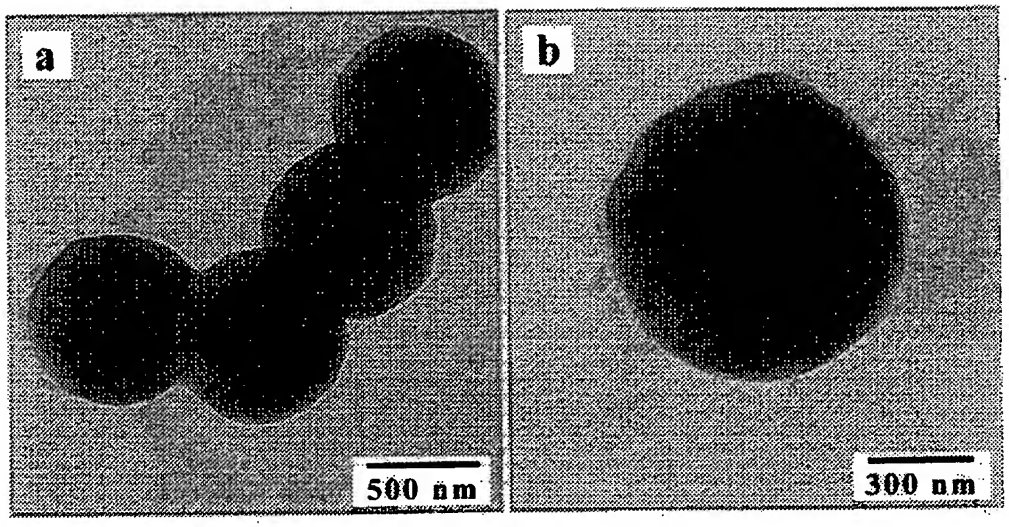


Figure 20



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/01854

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 B01J13/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 B01J A61K B05D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 667 148 A (HISAMITSU) 16 August 1995 (1995-08-16) claims 1-7	
A	EP 0 472 990 A (BAYER AG) 4 March 1992 (1992-03-04) claims 1-8	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 1999	21/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fouquier, J-P

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. ational Application No
PCT/EP 99/01854

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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